Modulation of the plasminogen system by thrombin activatable fibrinolysis inhibitor (TAFI)

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Ana Helena Canas Guimarães

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Modulation of the plasminogen system by thrombin activatable fibrinolysis inhibitor (TAFI)

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de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
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in het openbaar te verdedigen
ten overstaan van de promotiecommissie
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door

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geboren te Braga, Portugal

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copromotor: dr. D.C. Rijken

The studies presented in this thesis were performed at the Department of Biomedical Research of TNO Prevention and Health, Gaubius Laboratory, Leiden, at the Laboratory of Physiology of the Institute for Cardiovascular Research, Amsterdam and at the Department of Hematology of the Erasmus MC, University Medical Center Rotterdam.

"I am a part of all that I have met"

Alfred Lord Tennyson

Para o Rao, o meu anjo da guarda.

Contents

Modulation of the plasminogen system by TAFI

Discussion

Summary / Samenvatting

Abbreviations

α_2-AP	α_2-antiplasmin (*see also plasmin inhibitor*)
aa	amino acids
APC	activated protein C
APSAC	anisoylated plasminogen-streptokinase activator complex
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CAD	coronary artery disease
CABG	coronary artery bypass grafting
C/EBP	CCAAT/enhancer-binding protein
CPB	pancreatic carboxypeptidase B
CPN	carboxypeptidase N
CPR	carboxypeptidase R
CPU	carboxypeptidase U
CRP	C-reactive protein
DNA	deoxyribonucleic acid
DD(E)	complex of D-dimer non-convalently associated with fragment E
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
DSPA	desmodus rotundus salivary PA
DVT	deep venous thrombosis
εACA	ε-amino caproic acid
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylenediaminetetraacetic acid
FXIII	factor XIII
FCS	fetal calf serum
FbDP	fibrin degradation products
FII	prothrombin
FIIa	thrombin
FITC	fluorescein isothiocyanate
GEMSA	guanidinoethyl-mercaptosuccinic acid
GRE	glucocorticoid response element
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HepG2	human hepatoma cell line
hMVEC	human microvascular endothelial cell
HMw	high molecular weight
HPLC	high performance liquid chromatography
HS	human serum
IL-1	interleukin 1
IL-6	interleukin 6
IS	ischaemic stroke
K2tu-PA	Amediplase
LPS	lipopolysaccharide, bacterial
mRNA	messenger ribonucleic acid
MERGETPA	DL-2-mercaptomethyl-3-guanidino-ethylthiopropanoic acid
MI	myocardial infarction
MoAb	Monoclonal antibody
PA	plasminogen activator
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PC	protein C
PCI	potato carboxypeptidase inhibitor
plasma proCPB	plasma procarboxypeptidase B
PCR	polymerase chain reaction
PI	plasmin inhibitor
Plg	plasminogen
PNP	pooled normal plasma
PPACK	H-D-Phe-Pro-Arg-chloromethylketone
scu-PA	single-chain urokinase-type PA
SNP	single nucleotide polymorphism
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SD	standard deviation
SEM	standard error of the mean
STA	staphylokinase
TAFI	thrombin activatable fibrinolysis inhibitor
TAFIa	activated TAFI
TAFIai	inactivated TAFIa
TBS	Tris buffered saline
TF	tissue factor
TM	thrombomodulin
TNFα	tumor necrosis factor α
TNK-tPA	Tenecteplase
tPA	tissue-type PA
tcu-PA	two-chain urokinase-type PA
Tris	tris(hydroxymethyl)aminoethane
r-Hir	recombinant hirudin
r-tPA	recombinant tPA
RT-PCR	reverse transcriptase-polymerase chain reaction
UAP	unstable angina pectoris
uPA	urokinase-type plasminogen activator
3'-UTR	3'-untranslated region

INTRODUCTION

CHAPTER 1

Introduction

Thrombin activatable fibrinolysis inhibitor

Thrombin activatable fibrinolysis inhibitor (TAFI, EC 3.4.17.20) was discovered at the end of the 80s, when two groups identified independently an unstable carboxypeptidase present in serum preparations. They named it carboxypeptidase R [1] and carboxypeptidase U [2], respectively. Another group [3] was studying the mechanism of the antifibrinolytic action of thrombin in tissue-type plasminogen activator (tPA)-mediated plasma clot lysis. They spent the following years searching for the "de Fouw Factor", which was responsible for this effect. Still, this puzzle only started to be unveiled [4] when TAFI was identified as a contaminant during the purification of α_2-antiplasmin and named plasma procarboxypeptidase B [5]. Finally in 1995, the protein was rediscovered when seeking to explain the profibrinolytic effect of activated protein C (APC) [6]. The protein was named TAFI because it could be activated by thrombin and when active inhibited fibrinolysis.

TAFI has been suggested to circulate in a complex with plasminogen [5,7] but this seems to be based only on its affinity for plasminogen in a purified system and direct evidence is still missing. TAFI was shown to bind to plasminogen, which was immobilised on a Sepharose column and, using a BIAcore system, it was found that the affinity of TAFI for Lys-plasminogen was 10-fold higher than for Glu-plasminogen (Kd - 0.035 μM and Kd - 0.3 μM, respectively) [7].

TAFI is a basic procarboxypeptidase, which is synthesised in the liver and released into the circulation as a single-chain glycoprotein of 60 kDa (Fig.1). Four of the five potential N-linked glycosylation sites are located on the activation peptide with the carbohydrates accounting for about 20% of the zymogen total mass [5,8-10]. As a result, the activation peptide migrates as a broad band on SDS-PAGE, which is difficult to visualise with standard staining procedures such as Coomassie brilliant blue (Chapter 2). The N-glycosylation site located in the catalytic domain (Asn219) was also found in an unglycosylated form. This suggests that TAFIa exists in both a glycosylated and unglycosylated variant [9]. The disulphide pattern of TAFI, composed of three intrachain disulphide bonds, is homologous to that of pancreatic carboxypeptidase B (CPB) [9] as previously predicted [11]. Therefore the disulphide pattern of TAFI was unable to explain the difference in enzymatic stability between TAFIa and pancreatic CPB.

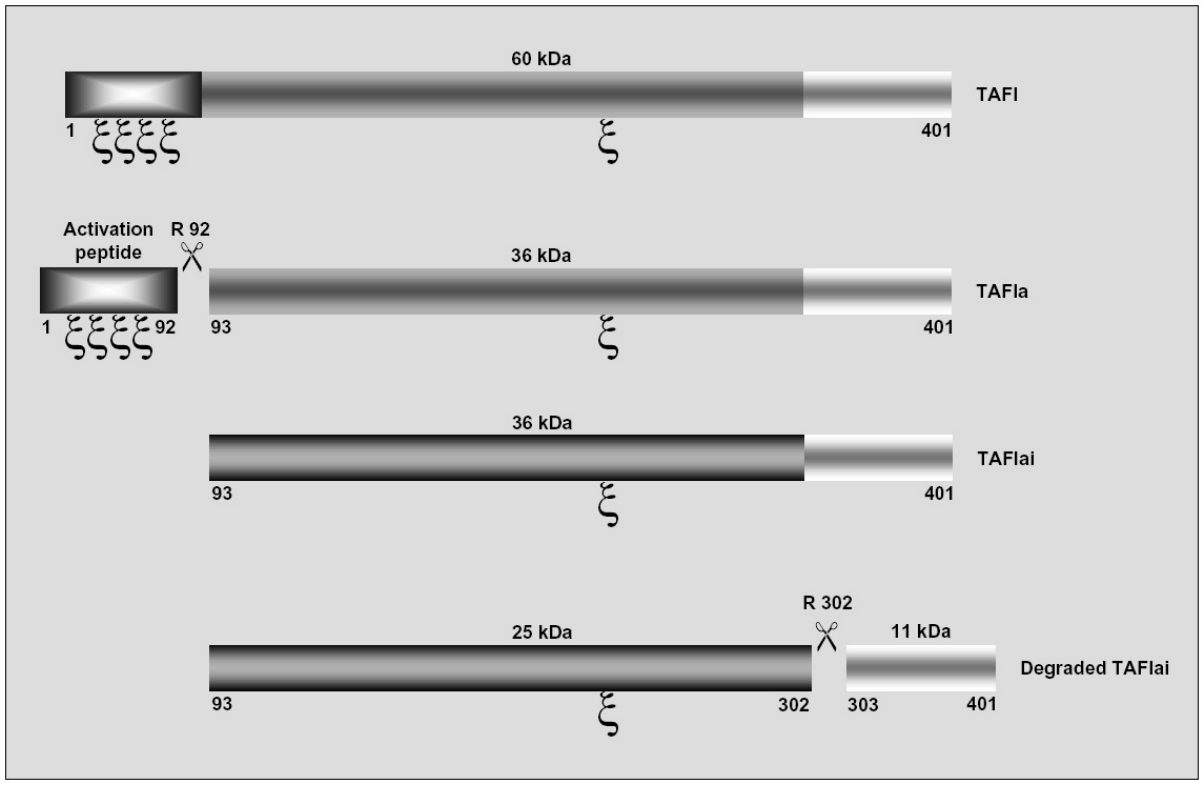

Figure 1. Schematic depiction of the activation and inactivation of TAFI. The proenzyme TAFI (60kDa) consists of 401 amino acids and contains 5 N-glycosylation sites located on the activation peptide (Asn22, Asn51, Asn63, Asn86) and on the catalytic domain (Asn219). Activation occurs by a single cleavage at Arg92 which results in the formation of TAFIa (36kDa) and release of the activation peptide. The complex between thrombin/thrombomodulin is seen as a likely activator in vivo though plasmin might also behave as a physiological activator of TAFI. Due to thermal instability TAFIa will undergo a conformational change and become inactive (TAFIai, 36kDa). This inactive form is in turn more susceptible to proteolytic cleavage at Arg302 by thrombin leading to the formation of degraded TAFIai (25kDa). Plasmin is capable of cleaving TAFI and TAFIa at additional sites (Arg327, Arg330) resulting in inactive forms.

TAFI has also been identified in platelets where it is stored intracellularly and seems to be synthesised during the intermediate and late stages of megakaryocytopoiesis [12]. This TAFI pool can be secreted upon platelet activation increasing the local TAFI concentrations within the fibrin mesh.

The proenzyme TAFI requires proteolytic cleavage at Arg-92 (Fig.1) in order to release the activation peptide and uncover the active site, generating the active enzyme (TAFIa, 36 kDa). Thrombomodulin (TM), a transmembrane glycoprotein receptor of thrombin required for the activation of protein C (PC) was found to work as a cofactor for thrombin in the activation of TAFI

[6,13,14]. When the complex between thrombin and thrombomodulin, in the presence of calcium [15], is formed the activation of TAFI and PC are enhanced 1250-fold [13] and 1000-fold [16], respectively. However, when PC is bound to the endothelial protein C receptor (EPCR), an additional stimulation of the activation by thrombin/thrombomodulin complex is accomplished (20-fold) [16]. The active enzymes, TAFIa and APC, have opposing effects on the haemostatic balance with one resulting in the down-regulation of fibrinolysis while the other down-regulates coagulation. The preference for TAFI or PC activation depends on the thrombomodulin concentration with low TM concentrations (< 5 nM) favouring TAFI activation and higher TM concentrations (~10 nM) leading preferentially to the activation of PC [17]. Interestingly, different domains of thrombomodulin are required for the activation of either TAFI or PC. Epidermal growth factor (EGF)-like repeats 4-6 of TM provide normal cofactor function for effective activation of PC by thrombin while TAFI in addition to the EGF-like domains 4-6 also requires a part of the EGF-like domain 3 [18-20]. Thrombomodulin has been detected in a variety of cells and tissues and TM expression is known to fluctuate on the endothelium according to the vessel size with larger vessels, such as arteries, bearing lower TM concentrations than smaller ones, such as capillaries. In addition, the presence of soluble TM in the circulation is used as a prognostic marker of endothelial cell membrane injury [21].

Activation of TAFI can also be mediated by trypsin or by plasmin by a single cleavage at Arg-92 in analogy to the activation mediated by thrombin [5,22]. The efficiency of TAFI activation by plasmin increases around 15-fold in the presence of heparin or other glycosaminoglycans (GAGs) [23]. These GAGs are synthesised by endothelial cells and are present in the extracellular matrix and on the cell surface [24].

The mechanism of TAFI in the inhibition of fibrinolysis

After the formation of a thrombus, the fibrinolytic system starts off by binding plasminogen and tissue-type plasminogen activator (tPA) to the fibrin mesh. This binding is mediated by specific interactions between internal lysines in fibrin and lysine-binding sites in plasminogen and tPA [25]. This first phase of plasmin generation slowly initiates fibrin degradation creating new C-terminal lysine residues, which during the second phase up-regulate plasminogen binding and plasmin generation [26,27]. TAFIa

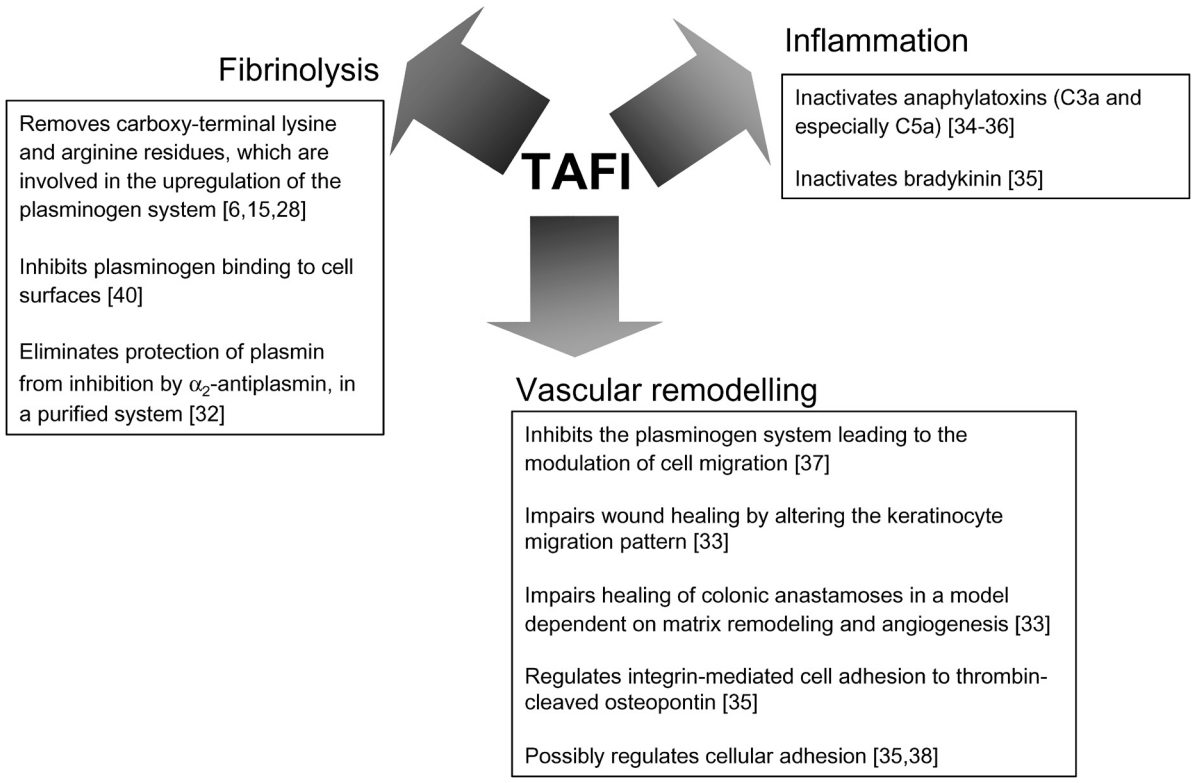

Figure 2. Schematic depiction of putative TAFI functions based on recent literature.

is able to hydrolyse these C-terminal lysines from partially degraded fibrin, restricting plasminogen binding and plasmin generation and thus inhibiting fibrinolysis (Fig.2) [6,15,28]. Correspondingly, treatment of fibrin degradation products with TAFIa reduced their cofactor activity in DSPA and tPA-mediated plasminogen activation [29]. Moreover, plasmin bound to C-terminal lysine residues is more resistant to inactivation by its naturally occurring inhibitors [30,31]. It has been shown in a purified system that TAFIa was able to abrogate this protection by removing these C-terminal lysines forcing plasmin to remain in solution where it is rapidly inhibited by α_2-antiplasmin [32].

Besides the role of these C-terminal lysine residues during thrombus resolution, C-terminal arginine and lysine residues were shown to mediate other extracellular protein-protein interactions, which are important during wound healing [33], control of inflammation [34-36], cellular migration [33,37], cellular adhesion [35,38] and in the regulation of cellular fibrinolysis [39] [40-43](Fig.2).

Regulation of TAFIa activity - role of stability and inhibitors

So far, TAFIa has no known physiological inhibitor and regulation of TAFIa activity occurs by a spontaneous temperature-dependent process, which results in a conformational change and in the generation of inactivated TAFIa (TAFIai). This has been schematically depicted in figure 1. Hence, TAFIa activity is highly sensitive to temperature with the half-life of TAFIa increasing from about 10 min at 37°C, 45 min at 30°C to stable at 0°C [8,10,44]. In addition, TAFIa is stabilised in the presence of a substrate or of a competitive inhibitor [45]. After TAFIa undergoes the conformational change it becomes more prone to proteolysis [7] by thrombin at Arg302 (Fig.1) [10,23,44]. Besides being able to activate TAFI (Arg92), plasmin is capable of inactivating TAFI and TAFIa by proteolytic cleavage at Arg327 and Arg330 [46]. Interestingly, epsilon-amino caproic acid (ε-ACA) is able to protect TAFI from plasmin-mediated cleavage at Arg327 and Arg330 but not at Arg92 [46]. Similarly, ε-ACA is also able to limit the proteolytic cleavage of TAFI by trypsin [7].

Given that TAFIa is a zinc-containing metallocarboxypeptidase [47], zinc-chelating agents [5], such as 1,10-phenantroline result in inhibition. Other inhibitors of TAFIa have also been described such as, ε-ACA, guanidinoethyl-mercaptosuccinic acid (GEMSA) and DL-2-mercaptomethyl-3-guanidinoethyl thiopropanoic acid (MERGETPA) [2,7,48,49]. Still, in most in vitro work and in in vivo animal experimental models, potato carboxypeptidase inhibitor (PCI, also referred to as PTCI and CPI) has been used as a specific TAFIa inhibitor. At the concentration used, PCI specifically inhibits TAFIa but not carboxypeptidase N (CPN), the other basic carboxypeptidase present in plasma [50]. CPN is constitutively active and shows a preference for C-terminal lysines over C-terminal arginines. On the contrary, TAFIa displays greater substrate specificity for C-terminal arginines [22]. Recently, PCI and GEMSA were demonstrated to be able to inhibit or enhance the antifibrinolytic activity of TAFIa depending on the concentration of inhibitor as well as the concentrations of tPA and TAFIa [45,51].

TAFI function - insights from animal models

Although the in vivo relevance of TAFIa as a regulator of fibrinolysis is not yet clearly established, convincing data are available from several animal studies showing that TAFIa inhibitors strongly improve thrombolysis [52-57].

The potential role for TAFI as a natural anti-inflammatory agent (Fig.2) by inactivating inflammatory mediators, such as the complement-derived inflammatory peptides C3a and C5a and bradykinin, has also been investigated. Both in vitro and some in vivo evidence is available that corroborates a role for TAFI in the regulation of inflammation [34-37].

Recently, TAFI knock-out mice have been generated by different groups [33,37,58]. These mice did not present a clear phenotype displaying normal embryonic development and normal life expectancy. The mice were fertile with pregnancies carried out to full term and no differences were found either in the haemostatic parameters or in liver and kidney functions. TAFI knock-outs were also subjected to a number of acute

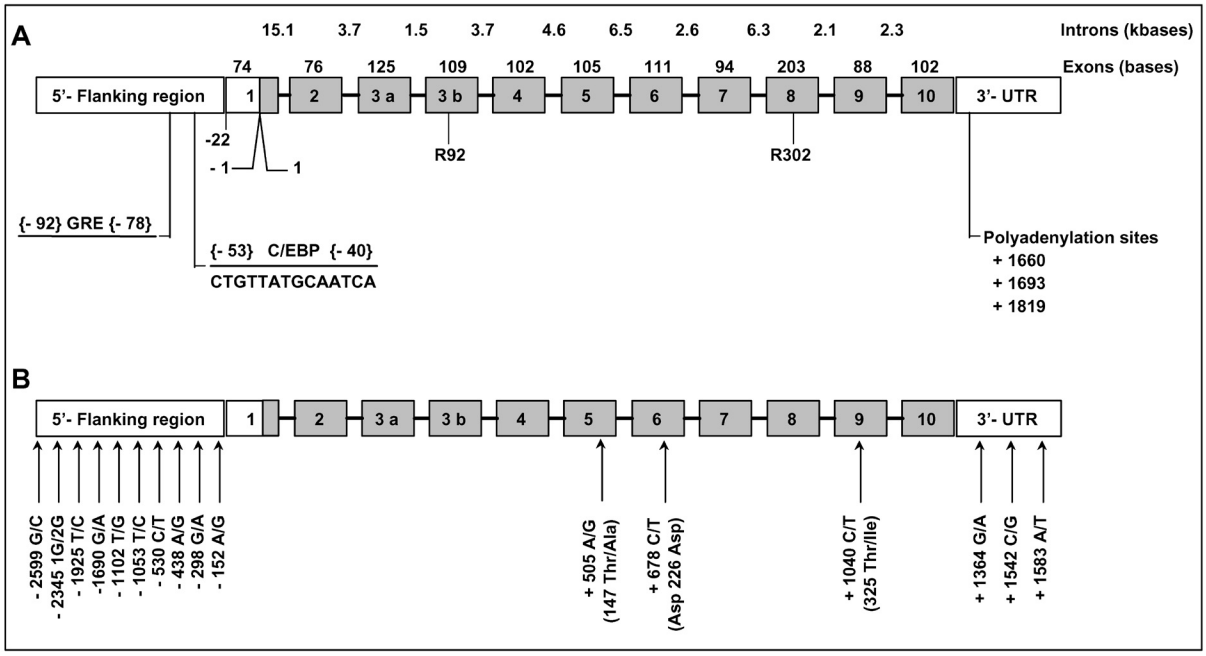

Figure 3. Schematic representation of the genomic organisation of the human TAFI gene and of the TAFI gene polymorphisms. **(A)** The TAFI gene is composed of 11 exons represented as numbered boxes (*length in bases*) separated by introns (*length in kbases*) [61]. The part in white on exon 1 represents the signal peptide, composed of 22 amino acids, which is removed intracellularly. The exons depicted in grey represent the sequences encoding for the TAFI protein with the activation peptide spanning from exon 1-3b, where the activation site (R92) is located. A putative C/EBP binding site has been identified between positions -53 and -40 of the promoter as well as a glucocorticoid response element (GRE) between nucleotides -92 and -78 [62]. **(B)** The location of TAFI polymorphisms identified in the promoter (*5'-Flanking region in white*) [68,69], in the coding region [70,71] represented by the exons and in the 3'-untranslated region (*3'-UTR*) [69]. The diagram allocates the SNPs identified in the TAFI gene.

challenges aiming to invoke a phenotype. Both models for venous and arterial thrombosis were employed with similar responses in knock-out and control animals. In addition, TAFI deficiency did not produce any advantage in thrombin-induced thromboembolism, factor X coagulant protein-induced thrombosis or endotoxin-induced disseminated intravascular coagulation and did not alter kaolin-induced disseminated intravascular coagulation. Notwithstanding, a recent paper [37] demonstrated a clear role for TAFI as a modulator of the plasmin(ogen) system in vivo by combining TAFI deficiency with a plasminogen heterozygous deficiency background. Of special interest was the fact that TAFI inhibited not only the fibrinolytic functions of plasminogen but also its cell migratory functions. This new role for TAFI as a modulator of cell-migrating and/or -invading functions seems to extend to other cell types. Another recent report has found a mild wound-healing defect in TAFI knock-out mice, which points to an involvement of TAFI in tissue repair processes [33].

Genomic organisation of the TAFI gene

The TAFI gene was mapped to chromosome 13q14.11 [59,60] and consists of 11 exons, spanning about 48 kb of genomic DNA [61] (Fig.3A). The TAFI promoter lacked a consensus TATA sequence resulting in the initiation of translation from multiple sites [61]. Three distinct polyadenylation sites were identified in the 3'-untranslated region (3'-UTR) of the TAFI gene [61] (Fig.3A).

The promoter sequences between nucleotides -141 and -73 were found to be crucial for liver-specific transcription [61]. In this region, a putative CCAAT/enhancer-binding protein (C/EBP) binding site was identified between positions -53 and -40 of the promoter as well as a glucocorticoid response element (GRE) between nucleotides -92 and -78 [62].

TAFI was shown to be an acute-phase protein in mice as injection of these animals with bacterial lipopolysaccharide (LPS) led to an increase both in TAFI plasma concentrations and in TAFI mRNA abundance in the liver [63]. By contrast, intravenous injection of LPS in healthy male volunteers brought about a decrease in TAFI antigen levels [64]. Still, in humans the plasma concentration of TAFI, C-reactive protein (CRP) and haptoglobin were correlated [65].

The effect of acute phase mediators on TAFI gene expression was investigated

in HepG2 cells [66]. IL-1 and IL-6 administration in combination induced a decrease in TAFI mRNA abundance, which was related to changes in the stability of the TAFI mRNA transcript. Alternative polyadenylation can result in rapid changes in transcript abundance by altering, for instance the mRNA stability. In agreement, the TAFI 3'-untranslated region was found to contain a cis-acting instability element that determines the stability of the TAFI transcripts [67].

TAFI gene polymorphisms

The TAFI gene has been scrutinised for single nucleotide polymorphisms (SNPs) in the promoter, coding and 3'-untranslated regions (Fig.3B). In the literature, nine SNPs were accounted for in the promotor (5'-flanking region) of the TAFI gene (-152 A/G; -438 G/A; -530 C/T; -1053 T/C; -1102 G/T; -1690 A/G; -1925 T/C [68] and -2345 2G/1G; -2599 C/G [69]) with several of these SNPs being localised in the proximity of transcription regulatory elements. One group of SNPs identified in the TAFI promotor, namely -1925 T/C, -530 C/T and -152 A/G, was in complete linkage disequilibrium (LD) while a second group of SNPs (-1690 A/G, -1102 G/T, -1053 C/T and -438 A/G) was in almost complete LD [68,69] In the coding region of the TAFI gene three SNPs have been reported in the literature, namely the +505 G/A (Ala147Thr), the +678 C/T (Asp226Asp) [70] and the +1040 C/T (Thr325Ile) [71]. Additionally, two SNPs were reported for the 3'-untranslated region (UTR) (+1542 C/G and +1583 A/T) [69].

Functional studies with four recombinant TAFI variants expressing either the residues 147Ala or 147Thr and either the residues 325Thr or 325Ile were carried out [72]. As expected from the previous characterisation of the TAFI 147Ala and 147Thr isoforms [70] no functional differences were found between variants containing this modification. In contrast, clear differences were observed between the TAFI Thr325Ile variants. The presence of an isoleucine at position 325 (TAFI 325Ile) nearly doubled the thermal stability of the active enzyme while the kinetics of activation of the isoforms by thrombin/thrombomodulin complex remained identical. Moreover, TAFIa 325Ile displayed a greater antifibrinolytic effect (30-60% increase) that arose from the increased ability of this isoform to release lysine residues from partially degraded fibrin. To date this SNP (1040C/T - Thr325Ile) comprises the only variation, which significantly alters the behaviour of TAFIa.

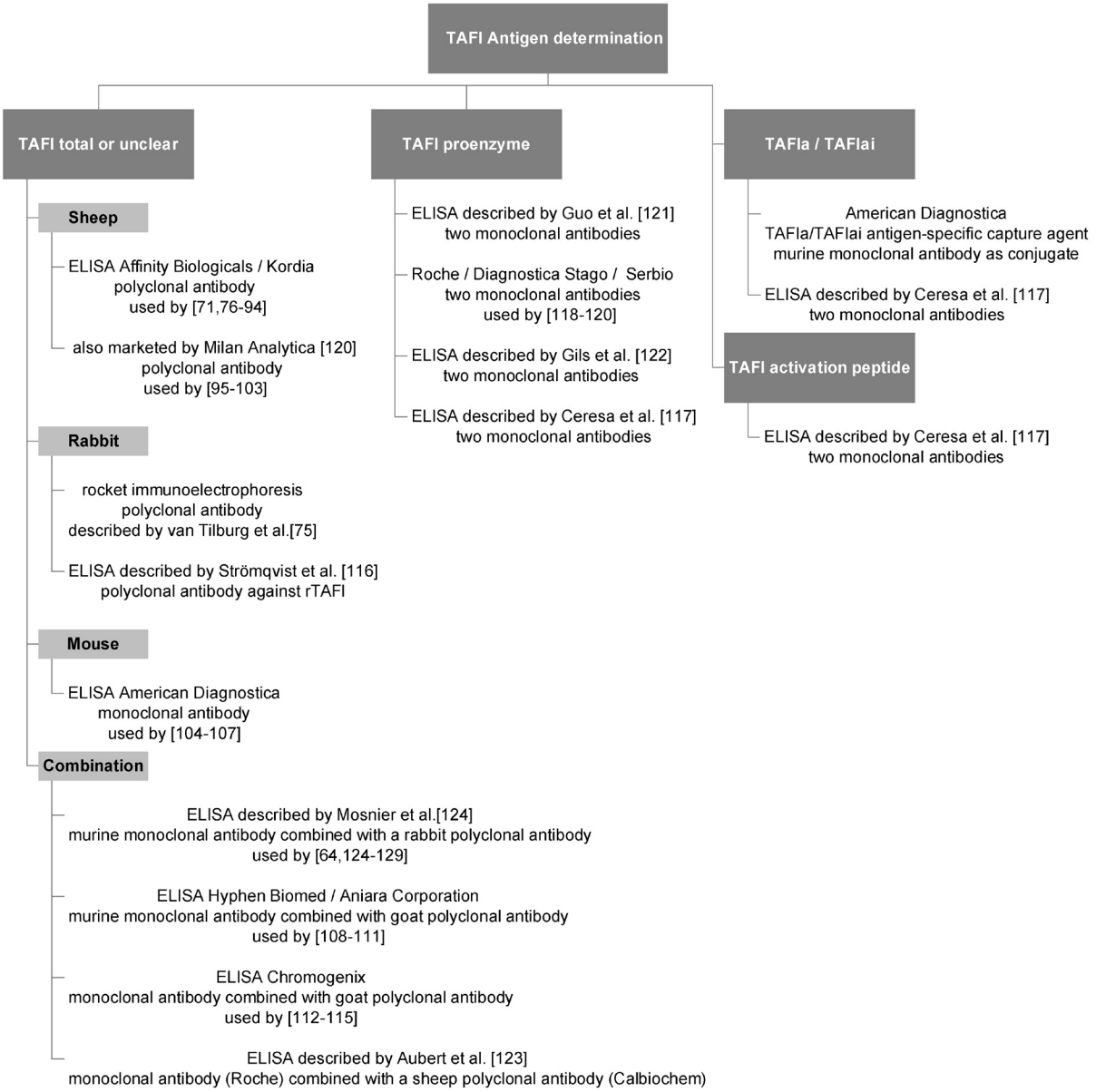

Figure 4. Schematic representation of the methods for antigen determination of TAFI. TAFI antigen levels can be determined in plasma using a variety of immunological assays with different principles. The figure contains information about product origin (commercial or developed in house), antibody reactivity and certain differences in antibody preparations (species immunised, polyclonal or monoclonal). Some of these assays were used in population-based studies and in such case references are provided.

Furthermore, information has been collected by distinct genome projects such as Ensembl [73] and HapMap [74] where several additional SNPs have been reported for the 5'- and 3'-flancking regions and coding region of the TAFI gene as well as for the intron regions. Additional silent SNPs

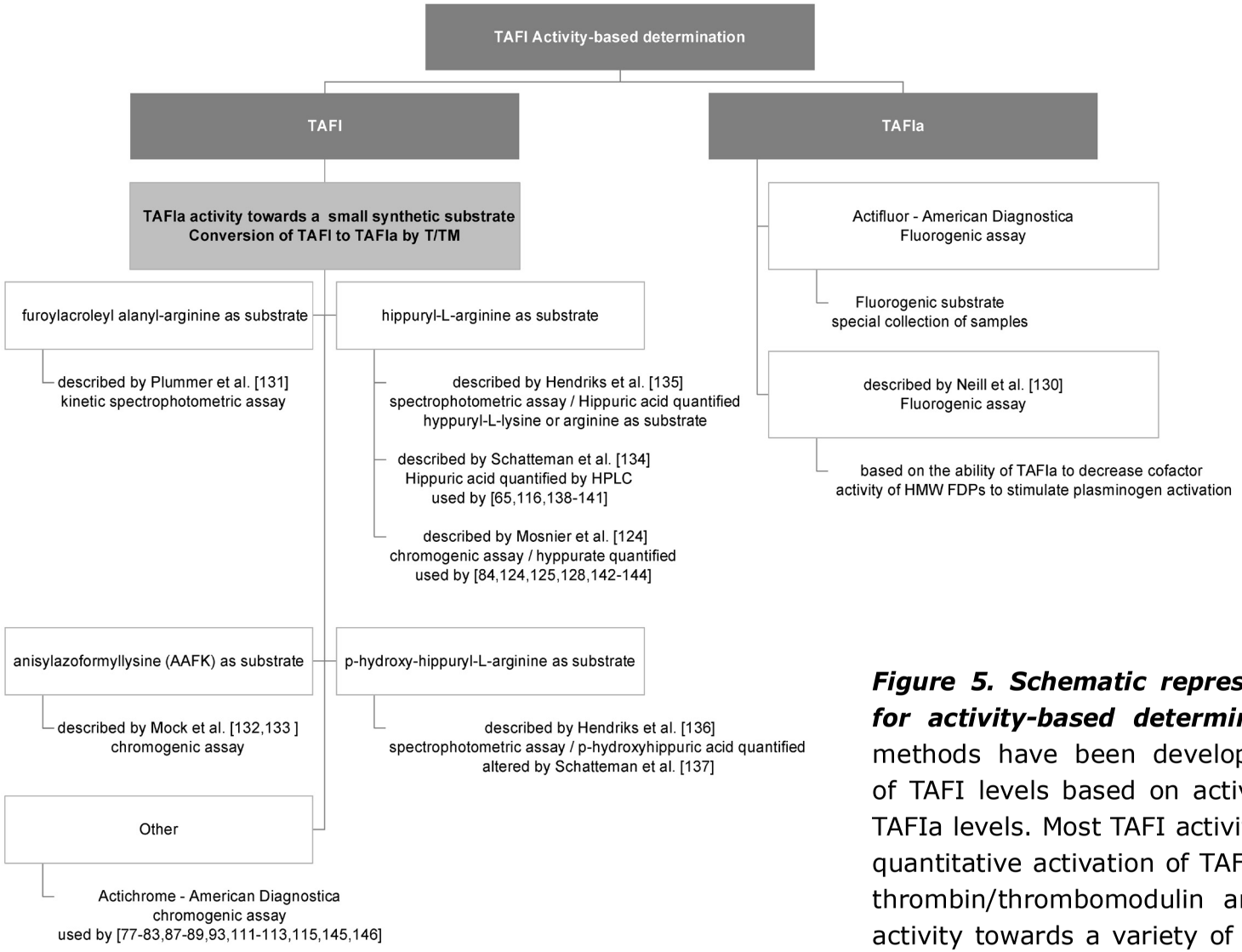

Figure 5. Schematic representation of the methods for activity-based determination of TAFI. Different methods have been developed for the determination of TAFI levels based on activity and more recently for TAFIa levels. Most TAFI activity assays are based on the quantitative activation of TAFI in the plasma sample by thrombin/thrombomodulin and determined the TAFIa activity towards a variety of substrates. Some of these assays were used in population-based studies and in such case references are provided in the figure.

(not shown Fig.3B) have been reported in the coding region of the TAFI gene (Asp97Asp, Asp142Asp, Pro221Pro, Ile251Ile). This information can be found on web-based databases.

Measurement of TAFI and TAFIa in plasma

Seeking to establish the role of TAFI as a possible causative agent of or risk marker for cardiovascular events resulted in the development of different methods for the determination of TAFI plasma concentrations. These methods are based on two different principles: determination of antigen levels or quantitative conversion of TAFI into TAFIa, usually by the thrombin/thrombomodulin complex, followed by the measurement of the basic carboxypeptidase activity generated. For both principles different approaches are employed resulting in subtle but important differences in the TAFI levels that are being determined (Fig.4 and 5).

Due to their straightforward methodology and regular implementation in the clinical set-up, enzyme-linked immunosorbent assays (ELISAs) are often selected for the determination of TAFI antigen levels although a rocket immunoelectrophoresis method has also been described [75] (Fig.4). The specificity of these assays for TAFI fractions in plasma ranges either from determination of total TAFI antigen (commercial: Affinity Biologicals [71,76-94] also marketed by Milan Analytica [95-103]; American Diagnostica [104-107]; Hyphen [108-111]; Chromogenix [112-115] or in-house [116] methods) to the determination of a particular fraction as for instance TAFIa/TAFIai [117], TAFI activation peptide [117] or just the TAFI proenzyme (commercial: Roche [118-120] or in-house [117,121,122] methods) (Fig.4). For some antigen assays the specificity has not been reported in the literature and therefore is marked as unknown (commercial: Calbiochem [123] or in-house [64,124-129] methods) (Fig.4).

Moreover, a variable immunoreactivity of some antibodies towards TAFI isoforms (Thr325Ile) has been demonstrated (Chapter 3 and [122]). For these antibodies the recognition of the TAFI 325Ile variant is impaired, leading to an underestimation in the determination of TAFI antigen levels of this variant and to a fictitious amplification of the genotype-related variation of TAFI concentration. This will be considered in more detail in the discussion of this thesis (Chapter 9). Nevertheless, using TAFI assays free of genotype-related artefacts combined with haplotype analysis the

estimation of the weight of TAFI gene polymorphisms on the TAFI antigen levels was found to be 25% [120].

The alternative is to determine TAFI plasma levels using an activity-based assay. Also in this case different assays have been developed to determine either TAFI after conversion into TAFIa or TAFIa activity in plasma (Fig.5). Recently, a fluorogenic assay was described for the determination of TAFIa activity in individual plasma samples [130]. This assay is based on the fact that TAFIa decreases the capacity of high molecular weight fibrin degradation products (HMW FDPs) to stimulate the plasminogen activation by DSPA and therefore, no correction is required for carboxypeptidase N (CPN). Another assay for the determination of TAFIa activity using a fluorogenic substrate for TAFIa has been made commercially available (Fig.5).

Other activity-based assays have been developed to exploit the specificity of TAFIa for carboxy-terminal lysines and arginines. Most of these assays rely on the conversion of TAFI into the active enzyme by thrombin/thrombomodulin followed by the determination of the activity of TAFIa in the hydrolysis of a small synthetic substrate (furoylacroleyl alanyl-arginine [131], anisylazoformyllysine [132,133], hippuryl-L-arginine [124,134,135], p-hydroxy-hippuryl-L-arginine [136,137] or unknown), as shown in Fig.6. These assays have been used in different epidemiological studies to evaluate the role of TAFI in cardiovascular disease (HPLC assay [138];[65,116,139-141] the chromogenic assay described by Mosnier et al. [84,124,125,128,142-144] and the Actichrome® assay [77-83,87-89,93,111-113,115,145,146]).

The TAFI functional isoforms 325Thr and 325Ile were shown to have similar activation kinetics by the thrombin/thrombomodulin complex as well as identical hydrolysis of a synthetic substrate (anisylazoformyllysine, AAFK) [72]. This means that activity assays relying on the cleavage of such a small synthetic substrate will probably detect both TAFI variants equally well. The activity-based assays determine the concentration of the proenzyme (TAFI), which can be converted into the active enzyme (TAFIa). It is imperative to bear in mind that these assays demand a quantitative and standardised conversion of the proenzyme to the active form and the assay conditions should take this into consideration because of the limited stability of TAFIa. In addition, correction for the interfering CPN activity is required.

TAFI functional activity has also been evaluated using other assays based on the ability of TAFIa to delay plasma clot lysis mediated by plasminogen activators (Chapter 6). The clot lysis time was shown to correlate with TAFI antigen [124]. However, these assays have as a downside the fact that other plasma components, such as plasminogen activator inhibitor-1 (PAI-1), α_2-antiplasmin, antithrombin and plasminogen, affect the clot lysis time resulting in an ambiguous measurement [127]. Notwithstanding, ways have been devised to circumvent this problem (Chapter 4).

The pathophysiological role of TAFI

The pathophysiological role of TAFI has been extensively studied. It is nowadays recognised that TAFI establishes the long sought-for link between coagulation and fibrinolysis whereas the implications for thrombotic diseases are still under discussion.
The ability of TAFI to cleave inflammatory mediators and inflammatory peptides allowing inflammation management has inferred an inhibitory role for TAFI during inflammatory processes. However, inflammation has another aspect, namely its potential to induce the activation of coagulation. In particular, if it were confirmed that TAFI is an acute phase response protein it would mean that in circumstances governed by inflammation-induced coagulation, TAFI might have a role in the development of a prothrombotic state.

In view of the antifibrinolytic function of TAFI, its involvement in thrombotic disease has been investigated. TAFI has been found to be a mild risk factor for venous thrombosis where increased TAFI antigen levels possibly lead to a hypofibrinolytic state. In contrast, in arterial thrombosis the results are conflicting. This is perhaps a consequence of the discrepancies in the TAFI assays (variable immunoreactivity of antibodies and/or deficient assay standardisation), which hamper interpretation of the results as well as the comparison between clinical data. These assays were also used to investigate the involvement of TAFI in other disease states such as diabetes, liver cirrhosis, kidney transplantation, nephrotic syndrome and dialysis. Efforts are now being made (Scientific Standardisation Committee of the ISTH) to compare assay performance and to achieve standardisation. This insight is essential for clarifying the pathophysiological role of TAFI.

The scope of this thesis

The aim of this thesis is to shed additional light on the molecular mechanisms of TAFI and to further unravel the physiological and pathophysiological relevance of TAFI.

The contents are structured into three sections. The first section deals with the biochemical properties of TAFI. The second section describes the assessment of TAFI assays along with the development, validation and implementation of new methodology for the functional determination of TAFI in plasma samples. And finally, the third section examines the involvement of TAFI in the modulation of the plasminogen system during fibrinolysis and during pericellular proteolysis.

Upon purification of TAFI from plasma, we studied the migration and detection of the activation peptide of TAFI during SDS-PAGE and characterised the reactivity of monoclonal antibodies against distinct TAFI fragments (Chapter 2).

TAFI antigen levels exhibit a large interindividual variability, in which genetic control seems to play a strong role. We evaluated the determination of TAFI antigen levels using different assays focusing on the relationship between TAFI antigen and TAFI genotype, particularly the Thr325Ile functional polymorphism (1040C/T) (Chapter 3).

The functional TAFI variant (TAFI Ile325Ile) exhibits increased stability but decreases expression levels thus reinforcing the importance of the determination of TAFI functional activity for studying the role of TAFI in cardiovascular disease. Consequently, we developed a new functional activity-based assay that was validated in a group of healthy individuals (Chapter 4). Thereafter, the role of TAFI in ischaemic stroke was studied by determining TAFI functional activity levels as well as the genotype distribution in a case-control prospective study (Chapter 5).

The efficiency of thrombolysis is determined to a certain extent by the properties of the PA (enzyme kinetic parameters, fibrin cofactor dependence), the thrombus structure (composition, degree of retraction) and by the supply of plasminogen and PA to the boundary and interior of the thrombus (transport of proteins, PA-fibrin clot-binding characteristics). Although TAFI is nowadays regarded as a potent inhibitor of fibrinolysis, its effect on the above-mentioned parameters, which influence not only in vivo fibrinolysis but also fibrinolytic therapy, is not fully resolved. Therefore, we

investigated plasma clot lysis mediated by increasing concentrations of a variety of PAs, which represent all major classes of thrombolytic drugs, under conditions in which lysis proceeds throughout the clot (internal lysis model) (Chapter 6). The inhibitory effect of TAFI was studied in this internal lysis model taking into consideration the role of the plasmin inhibitor. Furthermore, external plasma clot lysis, where lysis proceeds from the boundary to the interior of the plasma clot, was also studied using several models. In Chapter 7, the fibrinolytic efficacy of three PAs in these external lysis models was examined and the inhibitory effect of TAFI was evaluated.

In recent years, evidence suggesting the involvement of TAFI in other processes besides fibrinolysis and the ability of TAFI to behave as a broad modulator of the plasminogen system, is slowly growing. In Chapter 8, the involvement of TAFI in an in vitro model of capillary-like tube formation was investigated. This wound-healing angiogenesis model consists of a three-dimensional plasma clot matrix on top of which human microvascular endothelial cells (hMVECs) were seeded. The hMVECs upon combined stimulation with bFGF and TNF-α formed capillary-like structures. Due to the composition of the matrix, the results may be extrapolated to neovascularisation of a fibrinous exudate in vivo.

Finally, in Chapter 9 the results are discussed in association with recent insights from the literature in an attempt to provide a global view of the current path of TAFI research.

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THESIS

MODULATION OF THE PLASMINOGEN SYSTEM BY THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI)

BIOCHEMICAL PROPERTIES OF TAFI

CHAPTER 2

Migration of the activation peptide of thrombin activatable fibrinolysis inhibitor (TAFI) during SDS-polyacrylamide gel electrophoresis.

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Summary

Thrombin activatable fibrinolysis inhibitor (TAFI) is a plasma zymogen, which upon activation is capable of delaying fibrinolysis. We investigated the migration and detection of the activation peptide of TAFI during SDS-PAGE. Purified TAFI before and after activation by thrombin/thrombomodulin was electrophoresed on 4-20% polyacrylamide gels and stained with Coomassie Blue as well as Western blotting. Before activation, Coomassie Blue staining resulted in one main band of TAFI. After activation, a sharp band corresponding to TAFIa was observed. No distinct activation peptide was detected, in agreement with the literature. Western blotting using a polyclonal anti-TAFI antibody, on the other hand, showed one additional broad band with an Mr of about 33,000 after TAFI activation. N-terminal sequence analysis confirmed that this band represented the activation peptide of TAFI. In addition, we tested the reactivity of two anti-TAFI monoclonal antibodies (MA-T3D8 and MA-T18A8) towards TAFI before and after activation by Western blotting. Both monoclonal antibodies recognised TAFI. After activation of TAFI, MA-T3D8 reacted with TAFIa, while MA-T18A8 reacted with the activation peptide.

We identify the 33,000 band as the activation peptide of TAFI and exemplify the use of this information for the characterisation of monoclonal antibodies against TAFI.

Introduction

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a recently discovered plasma inhibitor of the fibrinolytic system [1,2]. The activated form of TAFI (TAFIa) is a carboxypeptidase B, which removes carboxy-terminal lysine and arginine residues from partially degraded fibrin. Because these residues represent binding sites of plasminogen, TAFIa reduces the fibrin-binding of plasminogen and thereby the activation of plasminogen and eventually fibrinolysis [3-5].

TAFI is a glycoprotein of 401 amino acid residues and has an Mr of about 60,000. The zymogen can be activated by thrombin, plasmin or trypsin, which cleave the protein between Arg-92 and Ala-93 [6]. The action of thrombin is strongly accelerated by thrombomodulin [7]. The activation results in a 92-amino acid activation peptide containing the four potential sites for N-glycosylation of TAFI and a 309-amino acid catalytic domain

with an Mr of about 36,000. TAFIa can be further degraded by thrombin at Arg-302, which results in two peptides with an Mr of 25,000 and 11,000, respectively [8,9]. A three-dimensional model of TAFI has been constructed on the basis of its homology with human pancreatic procarboxypeptidase B [10].

The activation peptide of TAFI has a theoretical Mr of 10,200 (without carbohydrate) and revealed after isolation by HPLC an Mr of 19,400 by mass spectrometry (with carbohydrate) [8]. The migration of the activation peptide during SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is not well documented and the literature about it is confusing.

Eaton *et al* [6] studied the activation of TAFI by using SDS-PAGE. They observed after prolonged incubation with trypsin a very faint band with an Mr of 15,000 and with the same amino-terminal amino acid sequence as the intact molecule, suggesting that the activation peptide migrated as a 15,000 protein. Bajzar *et al* [11] reported in their original paper on TAFI purification that SDS-PAGE of TAFI during a treatment with thrombin showed initially the appearance of 35,000 and 14,000 bands, followed by a 25,000 band. They suggested that TAFI was first cleaved to form 35,000 and 14,000 fragments. Amino-terminal sequence data of the 35,000 band showed, however, that the 35,000 band was composed of two unresolved proteins, with respective sequences corresponding to those of the 60,000 and the 25,000 proteins. The 14,000 band was not sequenced. Valnickova *et al* [12] studied the activation of TAFI by trypsin by using SDS-PAGE and also found two amino-terminal sequences in one sharp 36,000 band. They concluded that the activation peptide of TAFI co-migrated with TAFIa in the sharp protein band with an Mr of 36,000. Other investigators using either Coomassie Blue staining, silver staining, Western blotting or autoradiography of ^{125}I-labelled TAFI failed to show the generation of a distinct activation peptide on SDS-PAGE [8,9,13-15].

Our experiments establish the migration behaviour of the activation peptide of TAFI during SDS-PAGE and demonstrate that the peptide can be detected by Western blotting. This knowledge will facilitate biochemical studies on the TAFI protein. Furthermore, we report the use of our findings to localise the epitopes of monoclonal anti-TAFI antibodies on either TAFIa or the activation peptide.

Materials and Methods

Materials

Human thrombin was acquired from Sigma and rabbit lung thrombomodulin, with a specific activity towards thrombin of 1.2 units/μg, from American Diagnostica Inc. TAFI was isolated from plasminogen-depleted plasma by using ammonium sulphate precipitation, plasminogen-Sepharose chromatography and gel filtration on Superdex 200, essentially as described by Eaton *et al* [6].

Activation of TAFI

Purified TAFI (56 μg/ml - final concentration) was incubated at 22°C or 37°C in the presence of 5 mM $CaCl_2$, 2 unit/ml thrombin and 2 unit/ml thrombomodulin in 50 mM Hepes, 100 mM NaCl, 0.1 % (v/v) Tween 80, pH 7.5. The incubation was carried out for 30 min and subsequently quenched by addition of the thrombin inhibitor D-Phe-Pro-Arg chloromethyl ketone (PPACK - 1 μM). Samples were immediately used for SDS-PAGE.

Migration of the activation peptide of TAFI during SDS-PAGE

Precast 4-20% polyacrylamide Mini gels (iGels from Gradipore) were used for SDS-polyacrylamide gel electrophoresis. SDS was added to the samples containing TAFI before (0.22 μg) and after activation (0.44 μg) to a final concentration of 2 % (w/v). These were then heated for 2 min at 100°C. Samples and the standard proteins (low molecular weight kit from Pharmacia) were electrophoresed at 150 V for about 1 hour 30 min using the Laemmli buffer [16] system under non-reducing conditions. The gel was then stained with Coomassie Brilliant Blue.

Detection of the activation peptide of TAFI by Western Blotting

TAFI before activation (0.22 μg) and after activation (0.44 μg) as well as the activation components on its own were electrophoresed as described above. The gel was subsequently blotted onto nitrocellulose paper (Schleicher & Schuell) at 150 mA, overnight. Nonspecific binding sites were blocked by a 60 min incubation in block buffer (5% Nutrilon milk powder – Nutricia, in 7.5 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 145 mM NaCl pH 7.6). The blots were then incubated with a sheep anti-human TAFI HRP conjugated IgG (Affinity Biologicals - 0.5 μg/ml) in dilution buffer (20 mM Hepes, 150 mM NaCl, 1% (w/v) BSA, 0.1% (v/v) Tween 20, pH 7.2) for 120 min. After

several washings with dilution buffer the blot was stained with BM blue POD substrate (Roche). The blotted standard proteins were stained separately with Ponceau S (Helena Laboratories).

N-terminal protein sequence analysis

The N-terminal sequence analysis was performed by Edman degradation with an automated sequencer (Model 494 Procise, Applied Biosystems) [17]. The protocols, reagents and materials used were also from Applied Biosystems. Eurosequence bv (Groningen, The Netherlands) carried out the sequence analysis.

Reactivity of two Anti-TAFI monoclonal antibodies towards TAFI fragments

Two different anti-TAFI monoclonal antibodies (MA-T3D8 and MA-T18A8), which were generated by Gils *et al* [18] were used for the immuno-staining of TAFI (73 ng) before and after activation. SDS-PAGE and Western blotting were performed as described above. As the monoclonal antibodies were not HRP conjugated the immuno-staining protocol was altered. Briefly, the blots were incubated with MA-T3D8 (0.3 µg/ml) or MA-T18A8 (0.01 µg/ml) or both (MA-T3D8 - 0.3 µg/ml, MA-T18A8 - 0.01 µg/ml) in dilution buffer for 120 min. The blot was extensively washed with dilution buffer and incubated for 60 min with a secondary HRP-conjugated goat anti-mouse antibody (GAM/Ig/PO - Nordic) diluted 1:5000 in dilution buffer. After washing the blot was again stained with BM blue POD substrate (Roche).

Results

Migration of the activation peptide of TAFI during SDS-PAGE

TAFI before and after activation at 22°C was analysed by SDS-PAGE and stained with Coomassie Blue (Fig.1). Coomassie Blue staining revealed one main protein band of 65,000 before activation, and after activation a minor band of 65,000 representing some non-activated TAFI along with a major band of 37,000 of TAFIa. No distinct activation peptide was observed, in agreement with the literature.

Detection of the activation peptide of TAFI by Western Blotting

TAFI before and after activation at 22°C was also stained by Western blotting (Fig.1), showing in addition to the same protein bands seen with Coomassie Blue staining, a broad band with an Mr of about 33,000 for TAFI after activation. This band most likely represented the activation peptide. The slower migration than expected on the basis of its molecular weight and the heterogeneity are ascribed to its high carbohydrate content. The activation mixture was analysed under the same Western blotting conditions but no bands were observed (*not shown*). Moreover, a negative antibody control was also included, again without revealing any bands (*not shown*). The activation of TAFI was also performed at 37°C instead of 22°C (*not shown*). TAFI was fully activated and the activation peptide appeared again on the Western blot as a broad band of 33,000, now between two sharp bands of respectively, 37,000 (TAFIa) and 25,000 (further degraded TAFIa).

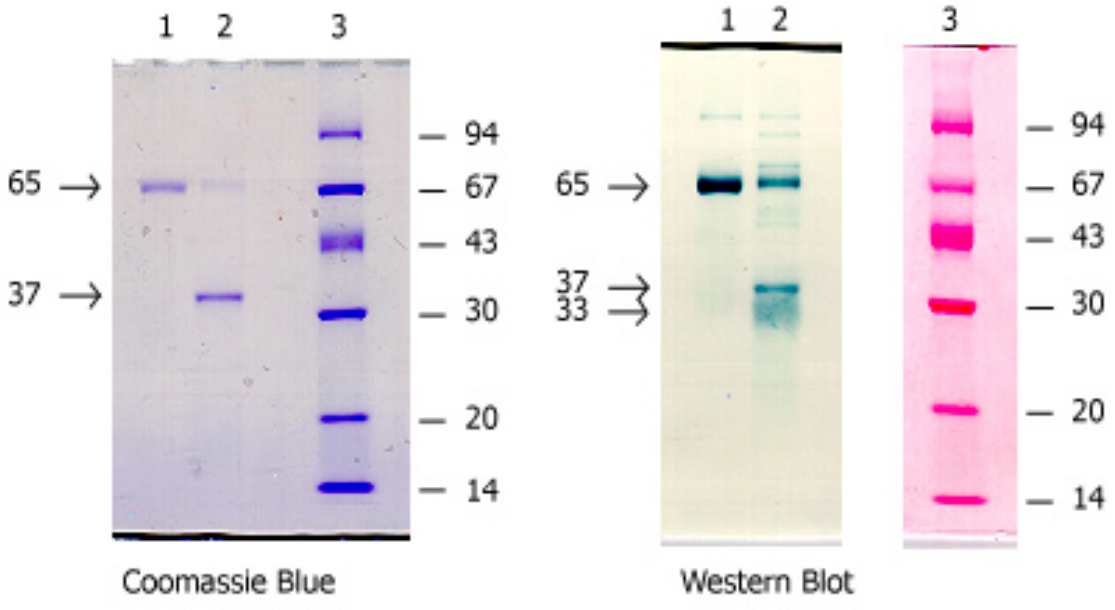

Figure 1. SDS-polyacrylamide gel electrophoresis of TAFI before and after activation with thrombin and thrombomodulin. TAFI before activation (*lane 1*) and after activation (*lane 2*) along with molecular mass standards (*lane 3*) were electrophoresed in parallel on two 4-20% gels under non-reducing conditions. One gel was stained with Coomassie Blue and the other one was blotted onto nitrocellulose paper and immuno-stained using sheep anti-human TAFI IgG conjugated to peroxidase and BM blue POD substrate. Positions of molecular mass standards are indicated on the right (10^{-3} x Mr).

N-terminal protein sequence analysis

In order to support the hypothesis that the 33,000 band represented in fact the activation peptide of TAFI, a gel slice below the 37,000 band was cut from the Coomassie Blue-stained gel and subjected to amino-terminal sequence analysis. The results showed a mixture of the following amino acid residues: (Phe/Ala) – (Ser?/Gln?) – (Ala/Ser?) – (Gly/Ser?) – (Tyr/Gln) – (Val/Tyr) – (Glu/Leu). These results were compatible with the presence of the activation peptide (amino-terminal sequence Phe-Gln-Ser-Gly-Gln-Val-Leu) and some material from the 37,000 band (TAFIa with amino-terminal sequence Ala-Ser-Ala-Ser-Tyr-Tyr-Glu) [6].

Reactivity of two Anti-TAFI monoclonal antibodies towards TAFI fragments

Two monoclonal anti-TAFI antibodies (MA-T3D8 and MA-T18A8) were used to inspect their reactivity towards TAFI and TAFI fragments generated by incubation with thrombin/thrombomodulin (Fig.2). Before activation, both the MA-T3D8 and the MA-T18A8 recognised one single band of 65,000, corresponding to TAFI. Upon TAFI activation, however, the band pattern obtained with these monoclonal antibodies was altered. The MA-T3D8

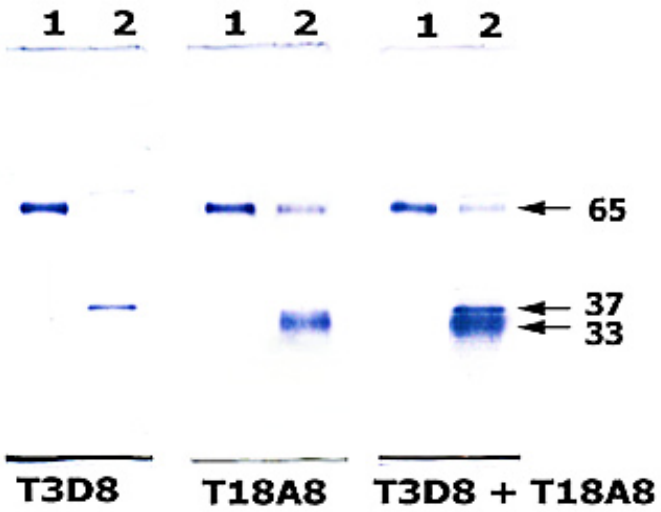

Figure 2. Western blot of TAFI before and after activation with two distinct monoclonal anti-TAFI antibodies. TAFI before activation (*lane 1*) and after activation (*lane 2*) with thrombin and thrombomodulin was electrophoresed under non-reducing conditions. The gel was blotted onto nitrocellulose paper and immuno-stained. Monoclonal anti-TAFI antibodies (MA-T3D8, MA-T18A8) were used as primary antibodies and HRP conjugated goat anti-mouse antibody as the secondary antibody. BM blue POD substrate was used for staining. Figures indicate 10^{-3} x Mr.

revealed a very minor band of about 65,000, which represented some non-activated TAFI, as well as a sharp band of 37,000, corresponding to TAFIa. In contrast, the MA-T18A8 recognised a broad band of 33,000 in addition to the minor band of 65,000. As we have shown, this broad band corresponds to the activation peptide. Combining both monoclonal antibodies resulted as expected in two distinct bands with an Mr of 37,000 (TAFIa) and 33,000 (activation peptide). The weaker staining of the minor band of 65,000 in the activated TAFI preparation by MA-T3D8 as compared to MA-T18A8 was due to the lower affinity of the former antibody (compare antibody concentrations in the Materials and Methods section).

Discussion

TAFI circulates as an inactive proenzyme. Upon activation, this carboxypeptidase B-like enzyme is cleaved between Arg-92 and Ala-93 resulting in a 92-amino acid activation peptide with four potential N-glycosylation sites. Several studies have characterised TAFI migration on SDS-PAGE. Nevertheless, the activation peptide of TAFI has never been detected on gels and information regarding it is somewhat confusing.

In order to study the migration and detection of the activation peptide during SDS-PAGE, we analysed once more purified TAFI before and after activation and used Coomassie Blue as well as Western blotting for staining (Fig.1). The Coomassie Blue staining pattern was very similar to that described in prior reports. TAFI before activation could be spotted as a relatively broad band of 65,000 with the same holding true for Western blotting. This probably reflected the heterogeneous glycosylation of TAFI [8,19]. Under moderate activation conditions, some non-activated TAFI could still be perceived on the Coomassie Blue and the Western blotting staining, as well as a sharp band of 37,000, which corresponded to TAFIa. Again, these results were analogous to previous data. Yet, we identified an additional broad band of about 33,000 on the Western blot. N-terminal sequence analysis of the polyacrylamide gel confirmed that this was in fact the activation peptide of TAFI with amino-terminal sequence Phe-Gln-Ser-Gly-Gln-Val-Leu. The migration of the activation peptide just ahead of TAFIa implies that the separation could be incomplete and that mixtures of TAFIa and the activation peptide could be found in sequence analyses [11,12]. In agreement with this, we found a second amino-terminal sequence Ala-Ser-Ala-Ser-Tyr-Tyr-Glu that matched TAFIa.

Our results explain most of the confusing data on the activation peptide in the literature. The high carbohydrate content probably prevents staining by Coomassie Blue and silver staining as well as the incorporation of ^{125}Iodine, making the activation peptide invisible in various experiments. In addition, the sort of gradient used for SDS-PAGE will influence the migration of the different TAFI fragments, particularly of the activation peptide due to its carbohydrate content.

On the basis of our findings we set out to inspect the recognition of TAFI and TAFI treated with thrombin/thrombomodulin by two different monoclonal anti-TAFI antibodies [18] (MA-T3D8 and MA-T18A8). Both monoclonal antibodies reacted with TAFI before activation. After the digestion of TAFI with thrombin/thrombomodulin, MA-T3D8 recognised TAFIa, a sharp band of 37,000 while MA-T18A8 recognised a broad band of 33,000, which as we have shown corresponds to the activation peptide. These results localise the epitopes of the monoclonal antibodies on the TAFI molecule and, simultaneously, confirm that the 37,000 and 33,000 bands represent distinct fragments of TAFI (i.e. TAFIa and the activation peptide, respectively).

The experiments described here establish the migration behaviour of the activation peptide of TAFI during SDS-PAGE and provide conditions for its detection by Western blotting. Moreover and as shown here this knowledge can aid in evaluating the reactivity of anti-TAFI antibodies towards TAFI fragments.

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THESIS | MODULATION OF THE PLASMINOGEN SYSTEM BY THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI)

ASSAYING TAFI

CHAPTER 3

Association between thrombin activatable fibrinolysis inhibitor (TAFI) genotype and levels in plasma. Comparison of different assays.

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Summary

TAFI antigen levels exhibit a large interindividual variability in which genetic control seems to play a major role. However, recent reports have questioned the association between TAFI concentration and genotype. These reports suggest that variable antibody reactivity towards TAFI isoforms, particularly the Thr325Ile polymorphism (1040C/T), may lead to artefacts in TAFI antigen levels. In order to compare assay outcome we determined plasma TAFI levels in 92 healthy individuals making use of an ELISA (commercial antibodies) and an electroimmunoassay (home-made antibodies) as well as a commercial chromogenic assay (Actichrome® TAFI). Each individual was genotyped for the - 438A/G and 1040C/T polymorphisms in the TAFI gene. We found a significant association of TAFI levels and genotype with both antigen assays and with the chromogenic assay. All assays displayed significant correlations with each other. Linear regression and Bland-Altman agreement analysis in the genotype sub-groups showed that neither the genotype nor the concentration affected the relationship between the Actichrome® TAFI and the Electroimmunoassay.

In contrast, the ELISA/Actichrome® TAFI and the ELISA/Electroimmunoassay relationships were concentration and genotype-dependent. Our results demonstrate that artefacts may arise when measuring TAFI antigen levels by ELISA. Nevertheless, the Electroimmunoassay and the Actichrome® TAFI support a genotype-related variation of TAFI concentration.

Introduction

Thrombin Activatable Fibrinolysis Inhibitor (TAFI) has been described as a potent fibrinolysis inhibitor that is present in plasma as a basic procarboxypeptidase, the active form of which, (TAFIa), can be formed during coagulation [1-3]. TAFIa can inhibit fibrinolysis by removing carboxy-terminal lysine and arginine residues from partially degraded fibrin leading to a decrease of plasminogen-binding sites on the fibrin surface [4-7]. Due to this, TAFI is also referred to as a link between coagulation and fibrinolysis. This confers an extra importance on the determination of TAFI plasma levels and activity as these may have both diagnostic and prognostic value for a number of diseases. Several reports have corroborated the involvement of TAFI in venous thrombosis [8-10], coronary artery disease [11-13], and myocardial infarction [14]. Although nowadays, several methods are

available for TAFI determination in plasma, evaluation and characterisation of the performance of these assays still remain a problem.

Several genetic polymorphisms have been identified in the TAFI gene and evidence has been gathered that points to a strong genetic control of plasma TAFI levels [15-17]. Recently, Brouwers *et al* [18] reported a new single nucleotide polymorphism (SNP), 1040C/T which is located in the coding region of the TAFI gene and results in the substitution of a threonine at position 325 by an isoleucine (Thr325Ile). This polymorphism was associated with TAFI antigen levels and in linkage disequilibrium with other TAFI SNPs. Simultaneously, Schneider *et al* [19] prepared TAFI variants with either a threonine or an isoleucine at position 325 and demonstrated that the Ile-325 variant had an increased stability and exhibited a greater antifibrinolytic effect than the Thr-325 variant.

TAFI plasma levels, in healthy individuals, have been shown to exhibit a large interindividual variability [15,17,20,21]. Recent reports have raised questions regarding the antibody reactivity of the different TAFI isoforms suggesting that the genotype-dependent variation of TAFI concentration may include assay artefacts (*unpublished observations*). The 1040C/T functional polymorphism seems to be especially involved. In order to evaluate assay agreement and to investigate genotype-related effects and possible assay-related artefacts, we compared three assays, i.e. Actichrome® TAFI activity assay, Electroimmunoassay and ELISA that rely on different methods. Actichrome® TAFI assay is a commercial chromogenic activity assay in which TAFIa is generated by thrombin/thrombomodulin. This chromogenic assay relies therefore on complete TAFI activation and on a similar activity of all TAFI isoforms towards the synthetic substrate. The Electroimmunoassay and the ELISA both depend on antibody reactivity, but are based on different principles and make use of different antibody preparations. We determined TAFI levels in citrated plasma from a group of 92 healthy individuals as well as the distribution of the - 438A/G and 1040C/T polymorphisms. We demonstrate that the TAFI concentration exhibits an association with TAFI genotype, but we emphasise that the magnitude of this association may be influenced by the choice of assay.

Materials and Methods

Subjects

A group of 92 healthy individuals composed of 46 males and 46 females with a mean age of 46.5 years, ranging from 21 to 75 years, was gathered. Blood was collected into 0.1 volume of 0.106 mol/l trisodium citrate. The blood was centrifuged at 2000*g* for 30 min at 4°C. The plasma was stored at - 80°C in aliquots of 0.4 ml. The participants gave informed consent.

Pooled normal plasma

Blood was collected by venipuncture in plastic tubes containing into 0.1 volume of 0.106 mol/l trisodium citrate. The blood was centrifuged at 2000 g for 20 min at 20°C. The donors consisted of 30 males and 40 females with a mean age of 38.7 years. The platelet-poor plasma of 70 healthy individuals (women on oral contraceptives were excluded) was pooled and used as a calibrator for the Electroimmunoassay and for the ELISA. The pooled normal plasma was considered to contain 1U/ml of TAFI.

TAFI Concentration (TAFI Actichrome®)

A commercial chromogenic assay (Actichrome® TAFI Activity kit - Lot. No. 022706 – instructions protocol October 2002; American Diagnostica) was used to determine the amount of activatable TAFI in plasma. This assay relies on the activation of TAFI by the thrombin/thrombomodulin complex to TAFIa, which then reacts with a chromogenic substrate. Plasma samples were diluted 25-fold in assay buffer. The diluted plasma sample (25 µl) was added to a well of a microtitre plate followed by 115 µl of an activation mixture containing thrombin, thrombomodulin and calcium ions or by 115 µl of assay buffer (unactivated plasma control). Both the activated sample and unactivated sample were incubated for 20 min at room temperature on an orbital plate shaker without a clot being observed. Then, 10 µl of an activation stop reagent was added to each well. During the 20-min incubation step serial dilutions of the TAFIa standard, which was supplied in the kit, were prepared. The TAFIa substrate (50 µl) was then added to the wells. The enzymatic reaction was followed in time by measuring the optical density at 405 nm while incubating at 25°C and shaking. The inter-assay variation coefficient was below 10% and the detection limit was 0.20 µg/ml TAFIa.

TAFI Antigen Concentration (Electroimmunoassay)

A rocket immunoelectrophoresis assay was used to determine TAFI antigen levels [8]. Briefly, 0.5% v/v of rabbit anti-TAFI IgG (6 mg/ml) was included in 1.0 % agarose (SeaKem LE, cat. No. 50 004; FMC BioProducts) gels in 31.6 mmol/l tricine, 91.5 mmol/l Trizma base, 1 mmol/l EDTA, pH 8.8. Standards (0.25-1.0 U/ml) were prepared by diluting the pooled normal plasma in TAFI-deficient plasma [8]. Standards and 2-fold diluted samples (in TAFI-deficient plasma) were applied (4.5 µl) in wells with a diameter of 2.5 mm. Plates were electrophoresed at 2 to 3 V/cm for 18 hours at 10°C to 15°C. After drying and staining the plates, the length of the precipitation peaks was measured and the TAFI concentrations computed from duplicate determinations. The Electroimmunoassay had an intra- and inter-assay variation coefficient of 6% and a detection limit of around 0.16 U/ml [8].

TAFI Antigen Concentration (ELISA)

A sandwich-type enzyme-linked immunosorbent assay (ELISA) was developed for the determination of TAFI antigen levels, using commercial sheep polyclonal antibodies against TAFI (Affinity Biologicals Inc.). The 100 µl of H_2SO_4 (1 mol/l) were added. The optical density was measured at 450 nm. TAFI plasma levels were calculated from duplicate measurements.

TAFI Genotyping

All individuals were genotyped for two previously identified polymorphisms, - 438A/G [16] and 1040C/T [18]. In order to determine the genotype of these two SNPs a duplex polymerase chain reaction (PCR) followed by a restriction fragment length analysis was performed. The following primer sequences were used: TAFI F4 (5′ CAG AGA TCC TGT AAT CAT CC 3′) and TAFI R2 (5′ CGC GAA GAC ATG CTG CTC AC 3′) for - 438A/G and TAFI-EX10-F (5′ TGC TTC CAG TCT CTA GTA GC 3′) and TAFI-EX10-R (5′ CAG TTG TAT TAC ATG TGA CC 3′) for 1040C/T. The PCR conditions used were: first denaturation step at 94°C for 4 min, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C, 2 min elongation at 72°C and finally 10 min at 72°C. The PCR products were digested with Hpy CH4 IV and SpeI, respectively, at 37°C for 4 hours and analysed on a 1.2 % agarose gel.

ELISA dilution profiles

Two separate plasma pools were prepared from individuals with the - 438 G/G, 1040 C/C (CC pool) or the - 438 A/A, 1040 T/T (TT pool) genotypes.

Each pool was composed of 7 healthy individuals, which belong to the subjects described above. Both pools included 4 men and 3 women, with a mean age of 44.7 and 45.3 years for the CC and TT pool respectively. The mean TAFI antigen concentration with the Electroimmunoassay was 1.37 and 0.94 U/ml for the CC and TT pool, respectively.

The pooled plasmas were diluted 5 to 1200-fold in dilution buffer and the TAFI ELISA was performed as described above. After addition of the TMB substrate, the development of the blue colour was followed in time by measuring the optical density at 620 nm every 30 s. The initial rates were obtained by linear regression analysis and expressed as delta $OD_{620\ nm}$ / time (min^{-1}) (mean of six repeats).

Statistical Analysis

Allele frequencies were calculated by gene counting and the genotype frequencies at both loci were in Hardy-Weinberg equilibrium (c^2 analysis with 1 df). The linkage-disequilibrium coefficient, D and the Lewontin's D' were estimated as outlined by Devlin and Risch [22]. Values are expressed as mean ± SD. One-way ANOVA was computed to look at the relation between TAFI levels and TAFI genotype. Pearson's correlation coefficients were calculated to study the associations between the assays (Actichrome® assay, ELISA and Electroimmunoassay). Linear regression analysis as well as Bland-Altman agreement analysis [23] were performed to evaluate the between-assay relationships in the entire sample group as well as in the 1040C/T genotype subgroups. ANCOVA was employed to test for differences in linear regression analysis for the between-assay relationships in the genotype subgroups, and an F test to verify if slopes were significantly non-zero. P values < 0.05 were considered statistically significant.

Results

TAFI Concentration and TAFI Genotyping

The genotype and allele frequencies of - 438A/G and 1040C/T TAFI single nucleotide polymorphisms were determined in 92 healthy individuals (Table I) and found to be in Hardy-Weinberg equilibrium ($P< 0.001$, c^2 analysis). The SNPs were, as previously described [18,24], in strong linkage disequilibrium ($D = 0.73$; $D' = 0.85$) and associated with the TAFI antigen levels, as shown in table I. Not only the immunologic assays

Table I. Distribution of - 438 A/G and 1040 C/T SNPs of the TAFI gene and association between TAFI genotypes and TAFI levels in 92 healthy individuals.

	N (%)	Actichrome® TAFI μg/ml (SD)	*P* *	Electroimmunoassay U/ml (SD)	*P* *	ELISA U/ml (SD)	*P* *
- 438 A/G							
AA	7 (8)	13.1 (3.0)		0.94 (0.15)		0.53 (0.17)	
AG	36 (39)	15.4 (2.6)	0.0002	1.09 (0.17)	0.0001	0.77 (0.19)	< 0.0001
GG	49 (53)	17.0 (2.4)		1.21 (0.16)		1.08 (0.29)	
1040 C/T							
TT (Ile/Ile)	8 (9)	13.5 (3.1)		0.98 (0.18)		0.56 (0.17)	
CT (Thr/Ile)	39 (42)	15.5 (2.5)	0.0004	1.10 (0.17)	0.0006	0.77 (0.18)	< 0.0001
CC (Thr/Thr)	45 (49)	17.1 (2.5)		1.21 (0.16)		1.12 (0.27)	
Overall	92 (100)	16.1 (2.8)		1.14 (0.18)		0.92 (0.30)	

**P* for relation between TAFI levels and TAFI genotypes calculated by ANOVA – post-test for linear trend.

Table II. Regression parameters obtained for each assay combination from linear regression analysis. The 92 healthy individuals were distributed in sub-groups according to the 1040C/T genotype, as shown in Fig.1.

	Electroimmunoassay / Actichrome® TAFI			ELISA / Actichrome® TAFI			ELISA / Electroimmunoassay		
1040 C/T	slope	intercept	r	slope	intercept	r	slope	intercept	r
TT (Ile/Ile)	0.046 ± 0.015	0.36 ± 0.21 *	0.594	0.0070 ± 0.022	0.46 ± 0.31 #	0.127	0.54 ± 0.31	0.026 ± 0.31 †	0.578
CT (Thr/Ile)	0.042 ± 0.009	0.45 ± 0.14 *	0.601	0.030 ± 0.011	0.30 ± 0.17 #	0.405	0.66 ± 0.14	0.035 ± 0.15 †	0.627
CC (Thr/Thr)	0.038 ± 0.008	0.55 ± 0.14 *	0.770	0.051 ± 0.015	0.22 ± 0.26 #	0.472	0.91 ± 0.22	0.014 ± 0.27 †	0.536
Overall	0.044 ± 0.005	0.43 ± 0.08	0.677	0.061 ± 0.010	- 0.06 ± 0.16	0.550	1.08 ± 0.14	- 0.32 ± 0.16	0.639

The Electroimmunoassay and the ELISA expressed in U/ml and the Actichrome® TAFI assay in μg/ml.

By ANCOVA: * $P = 0.249$. # $P < 0.0001$. † $P < 0.0001$.

(Electroimmunoassay and ELISA), but also the activity assay (Actichrome® TAFI assay) exhibited this association of genotype with concentration.

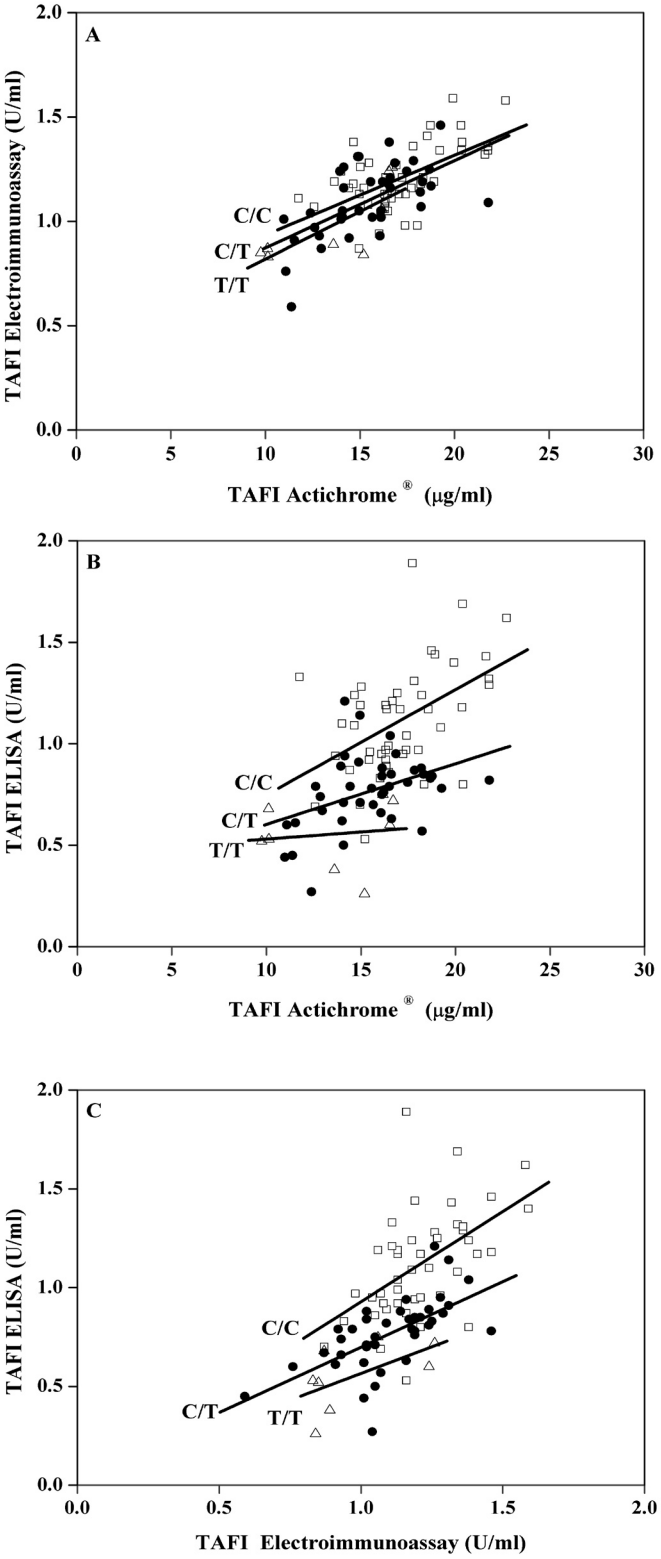

Figure 1. Effect of genotype on the between-assay relationships. TAFI concentration was determined with the Actichrome® TAFI activity assay, Electroimmunoassay and ELISA for a group of 92 healthy individuals. **A**, Electroimmunoassay/ Actichrome® TAFI assay. **B**, ELISA/ Actichrome® TAFI assay. **C**, ELISA/ Electroimmunoassay. (□) 1040 C/C, (●) 1040 C/T, (Δ) 1040 T/T. Depicted lines were obtained by linear regression analysis for each of the genotype sub-groups and table 2 provides detailed regression parameters.

Assay Evaluation by linear regression analysis

Statistically significant correlations were found both between the Actichrome® TAFI assay and the Electroimmunoassay ($r = 0.68$; $P < 0.0001$), between the Actichrome® TAFI assay and the ELISA ($r = 0.55$; $P < 0.0001$) and between the Electroimmunoassay and the ELISA ($r = 0.64$; $P < 0.0001$). Linear regression analysis revealed an intercept significantly different from zero for the relationships between the Electroimmunoassay and the Actichrome® TAFI assay and between the ELISA and the Electroimmunoassay (Table II).

To look for genotype-specific effects and/or possible assay-related artefacts we constructed plots of the Electroimmunoassay versus Actichrome® TAFI, ELISA versus Actichrome® TAFI and ELISA versus Electroimmunoassay for the 1040C/T-genotype sub-groups. Linear regression analysis, for each of these sub-groups, is shown in figure 1 and table II. For the Electroimmunoassay/Actichrome® TAFI plot we found that the regression lines obtained for the genotype sub-groups can be considered equal (fig. 1A) (by ANCOVA, $P = 0.249$). The ELISA/Actichrome® TAFI plot (fig.1B) and the ELISA/Electroimmunoassay plot (fig.1C) however, exhibit three distinct lines for the genotype sub-groups (by ANCOVA, $P < 0.0001$ and $P < 0.0001$, respectively). The results suggest that both the Actichrome® TAFI assay and the TAFI Electroimmunoassay are assays free of genotype-dependent artefacts. The ELISA on the other hand is dependent on the genotype and amplifies differences in TAFI concentrations in a genotype-related manner. This suggests distinct recognition of the TAFI variants in the two antigen

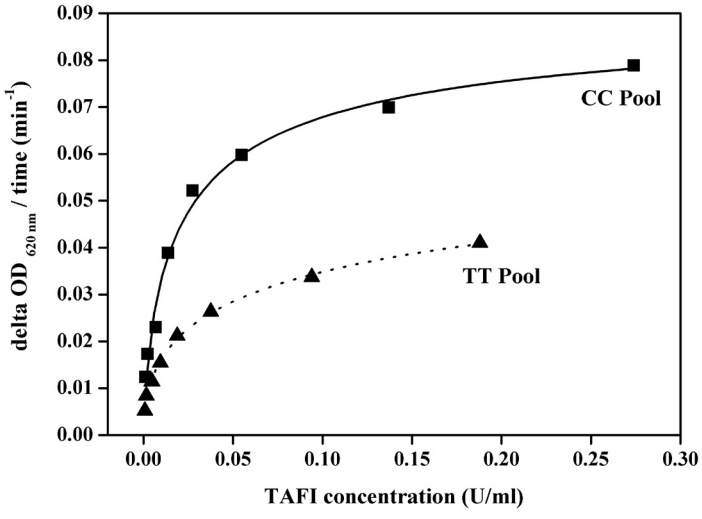

Figure 2. TAFI ELISA dilution profiles of the CC and TT pools (- 438G/G, 1040C/C and - 438A/A, 1040T/T respectively). The ELISA was performed as a kinetic assay and the delta OD 620nm / time plotted against the TAFI concentration obtained with the Electroimmunoassay (U/ml). (■) CC pool, (▲) TT pool. Each point results from six repeats.

assays, which could arise from the different IgG preparations or from the different principles of the immunologic assays.

Assay Evaluation by Bland-Altman agreement analysis

Bland-Altman difference plots were constructed from the results by plotting the differences between TAFI concentrations determined with two different assays against the average TAFI concentrations in these two assays (*not shown*). This statistical method can be used to assess agreement between two assays and is particularly useful when no reference method is available, as for TAFI determination.

For the whole group, concentration-dependent effects did not affect the agreement between the Electroimmunoassay and the TAFI Actichrome® (by F test, $P = 0.457$) while, for combinations involving the ELISA a concentration-dependent effect was encountered (by F test, $P < 0.0001$). Subsequently, Bland-Altman difference plots were constructed for the genotype sub-groups. Neither the 1040C/T genotype nor the TAFI concentration affected the agreement between the Electroimmunoassay and the TAFI Actichrome®. The agreement between the ELISA and the Electroimmunoassay or the TAFI Actichrome® however, was genotype-dependent and concentration-dependent.

ELISA dilution profiles

In order to study the ELISA response of the two TAFI variants in a greater detail, two different plasma pools (CC and TT pools) were serially diluted and tested in the ELISA. Figure 2 shows the delta $OD_{620\ nm}$ / time values plotted against the TAFI concentration in U/ml, as determined with the TAFI Electroimmunoassay and therefore not affected by the genotype.

The TT and CC pools showed distinct responses in the TAFI ELISA. The results suggest that the TT pool led to a lower maximal value than the CC pool, pointing to an impaired recognition of the 325Ile variant (- 438A/A, 1040T/T) in the ELISA.

Discussion

At present, it is not fully understood which TAFI single nucleotide polymorphism (SNP) or group of SNPs causes the high variability in TAFI levels and whether these can predict the risk of thrombosis. Several polymorphisms have been identified in the TAFI gene [10,16,18,25] and found to be in strong linkage disequilibrium. Recently, a SNP (1040C/T) was identified that results in an amino acid substitution at position 325 (Thr325Ile) [18]. Recombinant TAFI variants with either Ile325 or Thr325 were prepared and found to have a similar sensitivity to activation by the thrombin/thrombomodulin complex [19]. The hydrolysis of a synthetic substrate (anisylazoformyllysine - AAFK) by the activated TAFI variants was also identical. However, Thr325Ile is known to be a functional polymorphism with the variant Ile325 displaying enhanced stability and increased antifibrinolytic potential [19], but apparently lower antigen levels [18]. These opposing effects may hamper the interpretation of studies based solely on genetic association [24,26].

In the meanwhile, it was suggested that anti-TAFI IgG preparations might have a variable affinity towards different TAFI isoforms of the Thr325Ile SNP (1040C/T) (*unpublished observations*). If it would be confirmed that different IgG preparations or assays generate different results, the outcome of clinical studies may possibly depend on the assay methodology. This could aid the elucidation of the controversial results suggesting that high TAFI antigen in plasma is both protective against myocardial infarction [14,27] and angina pectoris [28], and a risk factor for venous [8-10] and arterial thrombosis [11-13]. Therefore, we set up a study to compare assay agreement in a group of 92 healthy individuals.

To do so, we selected three distinct assays to determine TAFI levels in plasma and genotyped the individuals. The results allowed us to discriminate between the outcome of the assays in the whole group and in the 1040 C/C, C/T and T/T sub-groups. We confirmed the significant association between the TAFI polymorphisms and plasma TAFI concentration for the two immunological assays (ELISA and Electroimmunoassay), although the genotype-dependence of the TAFI concentration was much more pronounced for the ELISA than for the Electroimmunoassay. Furthermore, we showed a similar association for the Actichrome® TAFI assay, which established that this association is not limited to immunological assays. It is relevant to stress at this stage that the Actichrome® TAFI assay relies

on the activation of the pro-enzyme and on the activity of TAFIa towards a synthetic substrate both of which seem to be identical for the TAFI 325Thr and TAFI 325Ile [19]. Hence, the Actichrome® TAFI assay is expected to detect the two TAFI variants equally well.

For the 92 healthy volunteers statistically significant correlations were encountered in all combinations of the assays. Furthermore, the relationships between both the Electroimmunoassay and the Actichrome® TAFI assay, and the ELISA and the Electroimmunoassay exhibited intercepts, which differed from zero. This may well arise from the fact that the Electroimmunoassay determines total TAFI antigen, which will comprise not only TAFI but also might include different TAFI fragments.

When the individuals where subdivided per genotype, a clear shift in response was seen for the relationships involving the ELISA. Regression analysis of the ELISA/Actichrome® TAFI and the ELISA/Electroimmunoassay relationships showed that the 1040 T/T sub-group resulted in the lowest regression line followed by the 1040 C/T and the 1040C/C sub-groups (fig.1). We confirmed these results with Bland-Altman agreement analysis where different responses were also encountered for the CC, CT and TT genotypes. The performance of the same analysis for the Electroimmunoassay/Actichrome® TAFI relationship produced identical responses. We concluded that these two assays are free of genotype-related artefacts while the ELISA involves a concentration and genotype-dependent effect that enhances erroneously the observed concentration differences between the genotype sub-groups. While the ELISA suggested that the TAFI levels in the C/C subgroup were 2-fold higher than in the T/T subgroup, the Electroimmunoassay and the Actichrome® TAFI indicated TAFI levels 1.2 to 1.3-fold higher in the C/C subgroup (Table I).

Dilution profiles of plasma pools composed of 1040C/C or 1040T/T individuals (Fig.2) confirmed that the response of the ELISA was variable for the two variants. The ELISA response for the pooled normal plasma used as calibrator was more similar to the CC pool than to the TT pool, due to the genotype frequencies (Table I). Altogether, this results in a variable estimation of TAFI concentration in individuals according to genotype but also according to the composition of the calibration pool.

After completion of this manuscript, Gils *et al* [29] described the development of a genotype 325-specific proCPU/TAFI ELISA. In addition, another combination of monoclonal antibodies was found to be completely

independent on the 325 genotype. The commercial antigen assays tested showed a variable dependence on the 325 genotype, which is in agreement with what we found using our ELISA. Furthermore, they completely attributed the genotype-related differences in TAFI concentration to the impaired reactivity towards the 325 genotype. This contrasts with our finding of a linear trend of TAFI concentration with the 325 genotype both for the Electroimmunoassay and for the Actichrome® TAFI assay. We believe that the authors have overlooked a small but significant genotype effect on TAFI levels that exists at least in human volunteers.

In conclusion, standardisation and better characterisation of the available TAFI assays and calibrators become increasingly important. We demonstrated that genotype-dependent artefacts might occur when measuring TAFI antigen levels by ELISA. As a result, one should exert caution in interpreting TAFI antigen levels given that comparison is only possible when the same method is used or when method characteristics are comparable. Moreover, it is also important to realise that as normal pooled plasma is usually used for ELISA calibration this may lead to additional discrepancies according to the composition of the pool. Nevertheless, based on the results obtained with the Electroimmunoassay and the Actichrome® TAFI assay, we support a genotype-related variation of TAFI concentration.

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CHAPTER 4

A new functional assay of thrombin activatable fibrinolysis inhibitor (TAFI).

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Summary

New TAFI assays are necessary for studying the role of this fibrinolysis inhibitor in cardiovascular disease. The identification of a functional SNP (1040C/T) leading to a TAFI-variant with increased stability but lower antigen levels has made the determination of functional activity even more essential. Therefore, we developed a new assay for the functional activity of TAFI in citrated plasma samples. This assay is based on the retardation of plasma clot lysis by TAFIa. TAFI activation was induced simultaneously with fibrin formation and lysis was mediated by rt-PA. The variability of other plasma components was minimized by a 20-fold dilution of the samples in TAFI-depleted plasma. Lysis times (-/+ potato carboxypeptidase inhibitor) and the TAFI-related retardation of clot lysis, the functional parameter of the assay, were determined in a group of 92 healthy volunteers, as well as TAFI antigen levels (Electroimmunoassay) and two TAFI SNPs (- 438A/G and 1040C/T). TAFI-related retardation was 19.8 ± 5.6 min (mean ± SD) and was correlated with the antigen level. The specific antifibrinolytic activity of TAFI was associated with the - 438A/G and 1040C/T genotypes. Individuals with the 325Ile-variant had on average a 34% higher TAFI-specific antifibrinolytic activity than individuals with the 325Thr-isoform. The TAFI-related retardation in the two groups of individuals did not differ, as a lower level compensated for the higher specific antifibrinolytic activity of the 325Ile-isoform. This assay provides valuable information about the performance of different TAFI isoforms and constitutes a new method for studying the role of TAFI in cardiovascular disease.

Introduction

Thrombin activatable fibrinolysis inhibitor (TAFI) or plasma procarboxypeptidase B was discovered about a decade ago and constitutes a connection between the clotting and fibrinolytic pathways [1-3]. Proteolytic cleavage of TAFI after Arg-92 by thrombin, plasmin, trypsin or the thrombin-thrombomodulin complex forms the active enzyme (TAFIa) and the glycosylated activation peptide [4-6]. The antifibrinolytic effect of TAFIa in vitro is dependent on the concentration of TAFI [7,8] as well as on the type and concentration of the plasminogen activator used [9]. TAFIa removes C-terminal arginine and lysine residues present on partially degraded fibrin [7,10-12]. These residues have a pivotal role in plasminogen activation and in the lysis of a plasma clot. Consequently, TAFIa inhibits clot lysis.

There is evidence that a single nucleotide polymorphism (SNP) in the TAFI gene, which results in the substitution of a Thr by an Ile at position 325 of the protein [13], is located in a region that is related to the thermal stability of TAFIa and therefore to its antifibrinolytic potential. Hence, an Ile at position 325 in recombinant TAFI doubles its stability (half-life at 37°C increases from 8 min to 15 min) and results in an increased antifibrinolytic activity [14]. However, the same SNP (1040 C/T) was found to be associated with TAFI antigen levels in plasma with the T allele (which codes for the 325Ile-isoform) being linked to lower antigen levels [13]. This probably arises from the fact that this SNP is in strong linkage disequilibrium with several other SNPs situated in the promoter region of the TAFI gene [13,15-17]. Indeed, the increased stability and activity of this isoform and its decreased antigen level pose a problem for the interpretation of results of TAFI assays.

Different methods have been employed for the determination of TAFI activity in plasma and recently, an assay was devised for the determination of endogenous TAFIa activity in plasma [18]. Some of the TAFI activity assays used clot lysis times [8,19,20]. However, most TAFI activity assays are based on the activation of TAFI zymogen and subsequent detection of TAFIa via the enzymatic cleavage of a small synthetic substrate [3,21-24]. Our aim was to develop an assay for the measurement of TAFI that would include TAFI activation, the limited stability of TAFIa as well as the removal of C-terminal lysines and arginines from partially degraded fibrin by TAFIa. This functional assay is based on the ability of activated TAFI to delay the lysis of a plasma clot mediated by r-tPA. A 20-fold dilution of the sample in TAFI-depleted plasma reduced the influence of other individual plasma components on lysis, which previously posed a problem for the specificity of lysis-based TAFI assays [20]. In addition, this predilution assured the measurement of TAFI activity in a concentration range, which produced a proportional increase in response. By determining TAFI antigen levels as well as genotyping for the functional TAFI SNP (1040C/T – Thr325Ile) and a SNP in the promoter region (- 438A/G) we were able to show that the response of the TAFI functional assay is dependent not only on the TAFI level but also on the stability and activity of the particular TAFI isoform.

In our view this novel TAFI functional assay can be used for the determination of potential TAFI activity in a variety of individual plasma samples and may be used for the identification and characterisation of TAFI molecules with altered activity as well as for testing new pharmaceutical approaches for the modulation of the antifibrinolytic activity of TAFIa.

Materials and Methods

Materials

r-tPA (Actilyse) was kindly supplied by Boehringer Ingelheim (Ingelheim, Germany). Human thrombin was purchased from Sigma (St Louis, MO, USA) and both potato carboxypeptidase inhibitor (PCI) and human recombinant plasmin activator inhibitor 1 (PAI-1) were obtained from Calbiochem (La Jolla, CA, USA). For stability reasons, dilutions of the PAI-1 stock were performed in 20 mM Hepes, 1 mM EDTA, pH 6.5. Rabbit lung thrombomodulin (TM), with a specific activity towards thrombin of 1.2 units/µg, was acquired from American Diagnostica Inc (Greenwich, CT, USA). Plasmin inhibitor (previously α_2 –antiplasmin) and fibrinogen were obtained from Biopool (Umeå, Sweden) and Enzyme Research Laboratories (South Bend, IN, USA), respectively. Plasminogen was purified from human plasma by lysine-Sepharose chromatography [25]. All the other reagents were from Merck (Darmstadt, Germany).

Pooled normal plasma

Blood was collected by venipuncture from 70 healthy individuals (30 men, 40 women, mean age 38.7 years) in plastic tubes containing 0.1 vol of 0.106 M trisodium citrate. Blood was centrifuged at 2000*g* for 20 min at 20°C. Women on oral contraceptives were excluded. The platelet-poor plasmas were pooled and used as calibrator for the Electroimmunoassay [24,26]. The pooled normal plasma was considered to contain 1U/ml of TAFI.

Subjects

A group of 92 healthy individuals (46 males and 46 females, mean age of 46.5 years, range 21 to 75 years) was recruited among hospital personnel and their families. All subjects gave informed consent. Blood was collected into 0.1 vol of 0.106 M trisodium citrate and centrifuged at 2000*g* for 30 min at 4°C. The plasma was stored at - 80°C in aliquots of 0.4 ml.

TAFI-depleted Plasma

TAFI-depleted plasma was prepared using an anti-TAFI IgG sepharose column as previously described by Van Tilburg *et al* [26]. Briefly, rabbit polyclonal anti-TAFI antibodies were coupled to CNBr-activated Sepharose-4B (Amersham Pharmacia Biotech, Uppsala, Sweden) as described by the

manufacturer. Pooled normal plasma prepared from blood collected on citrate-phosphate-dextrose was applied to the anti-TAFI IgG sepharose column (10 mg IgG/mL sepharose) and the breakthrough fractions were tested for TAFI with a TAFI ELISA [24] and with the TAFI functional assay (see under). TAFI-depleted fractions were pooled and dialysed against 50 mM Hepes, 100 mM sodium chloride, 20 mM sodium citrate (pH 7.4), in order to remove phosphate ions. The plasma was then aliquoted and frozen at -20°C. TAFI-depleted plasma was used to dilute samples both in the TAFI functional assay and in the Electroimmunoassay.

TAFI Functional Assay

Individual citrated plasma samples were diluted 20-fold in TAFI-depleted plasma (a single batch of TAFI-depleted plasma was used to determine all individual samples). The diluted plasma samples (100 µl) were added to the wells of a microtitre plate containing 25 µl of a reaction mix. The mix was composed of thrombin (3.3 NIH units/ml), TM (0.6 units/ml), r-tPA (0.10 µg/ml), $CaCl_2$ (20 mM) and PCI (30 µg/ml where stated) in 50 mM Hepes buffer, pH 7.4 containing 0.1% w/v BSA. The concentrations between brackets refer to the final concentrations in the clotted plasma, which have been optimised as described previously [9]. The wells were immediately covered with paraffin oil (Merck – No.107162) and the microtitre plate was placed in the prewarmed (37°C) incubation chamber of a TECAN Sunrise® Microplate-reader (Giessen, The Netherlands). The optical density at 405 nm was monitored every minute for 150 min. A control with PCI was used for each sample. Lysis time (LT) was defined as the time point corresponding to a 50% decrease in optical density and a sigmoidal regression was used for its determination. The TAFI-related retardation was determined for each sample by subtracting the lysis time in the presence of PCI (LT_{+PCI}) from the lysis time in the absence of PCI (LT_{-PCI}). Using this assay the intra- and inter-assay variability of the TAFI-related retardation of pooled normal plasma (20-fold diluted) were 7 % and 13 %, respectively (n = 30; using several batches of TAFI-depleted plasma).

TAFI Functional assay: dilution profile

Serial dilutions of citrated plasma in TAFI-depleted plasma were prepared and the TAFI-related retardation of clot lysis was measured as outlined above. The dilution profile was inspected for pooled normal plasma and for citrated plasma from two individuals who were homozygous for the - 438 A/G and 1040 C/T TAFI polymorphisms. One individual had the - 438 GG, 1040 CC

genotype and the other the - 438 AA, 1040 TT genotype.

TAFI antigen levels (Electroimmunoassay) and genotyping

Rocket immunoelectrophoresis [27] was used to measure TAFI antigen levels [26]. The Electroimmunoassay and genotyping were performed as described previously [24]. The Electroimmunoassay had an intra- and inter-assay variation coefficient of 6% and a detection limit of around 0.016 U/ml [26]. The distribution of the individuals in the genotype subgroups according to gender was normal.

Statistical Analysis

Values are expressed as mean ± SD or mean ± SEM as indicated. The normality of the distribution of each functional assay parameter was tested in the entire group and in the genotype subgroups using both parametric and nonparametric tests. One-way ANOVA with a post-test for linear trend was used to look at the relation between TAFI levels and the TAFI genotype. Pearson's correlation coefficient was calculated to study

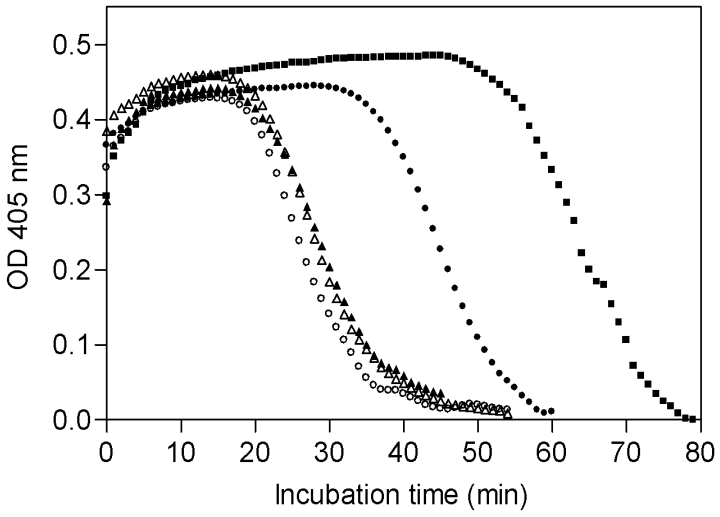

Figure 1. Clot lysis profiles obtained by plotting the OD 405 nm against the incubation time (min) (*typical example*). TAFI in each plasma sample was activated during clotting with thrombin (3.3 NIH units/ml), thrombomodulin (0.6 units/ml) and $CaCl_2$ (20 mM) in the presence of r-tPA (0.10 µg/ml). PCI was included (30 µg/ml), where stated. Closed squares, normal plasma. Closed circles, normal plasma diluted 20-fold in TAFI-depleted plasma. Open circles, normal plasma diluted 20-fold in TAFI-depleted plasma with PCI. Closed triangles, TAFI-depleted plasma. Open triangles, TAFI-depleted plasma with PCI. The baseline OD value was subtracted for each curve (about 0.4).

the associations between the TAFI functional assay parameters and the TAFI Electroimmunoassay. Linear regression analysis was performed to evaluate the between-assay relationships in the TAFI genotype subgroups. Multivariate regression analysis was performed to assess the effect of TAFI levels and TAFI genotypes considered together. *P* values < 0.05 were considered statistically significant.

Results

TAFI-depleted plasma

A typical example of the clot lysis profiles obtained is shown in Fig.1. The lysis times (LT) of TAFI-depleted plasma in the absence and presence of PCI were essentially identical (30.2 ± 3.6 min and 31.6 ± 4.0 min, respectively, mean ± SD, n = 25 with two batches of TAFI-depleted plasma). Normal plasma diluted 20-fold in TAFI-depleted plasma showed a 1.5-fold prolonged lysis time, which was abolished by the addition of PCI. Undiluted normal plasma showed a 2-fold prolonged lysis time. Figure 1 also demonstrated that the

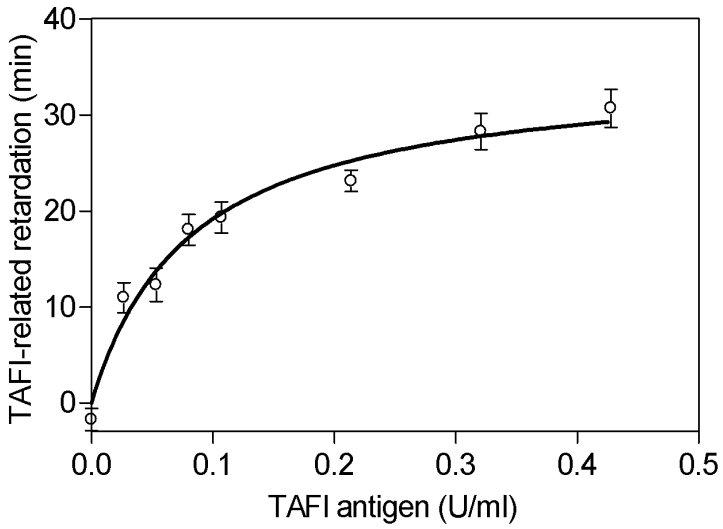

Figure 2. Dilution profile of pooled normal plasma in the functional assay of TAFI. Variable TAFI concentrations were obtained by performing serial dilutions of normal plasma in TAFI-depleted plasma. TAFI in each plasma sample was then activated during clotting with thrombin (3.3 NIH units/ml), thrombomodulin (0.6 units/ml) and $CaCl_2$ (20 mM) in the presence of r-tPA (0.10 µg/ml). Each sample was measured in the absence and in the presence of PCI and TAFI-related retardation was plotted against the TAFI antigen concentration. Each point represents the mean of seven independent experiments and bars indicate SEM.

levels of other constituents of plasma essential for clot formation and lysis were not appreciably altered by the immunoadsorption procedure, as the $LT_{+\ PCI}$ of the plasma remained constant before and after TAFI depletion.

Dilution profile

From serial dilutions of pooled normal plasma in TAFI-depleted plasma we obtained the dilution profile for the TAFI-related retardation, shown in Fig.2. Increasing TAFI concentrations produced an increase in retardation up to about 0.20 U/ml. At higher TAFI concentrations a plateau in the response was reached.

TAFI functional assay

On the basis of the dilution profile of pooled normal plasma (Fig.2) we used a 20-fold dilution of plasma samples in TAFI-depleted plasma in order to determine TAFI-related retardation in a relatively sensitive range. Lysis

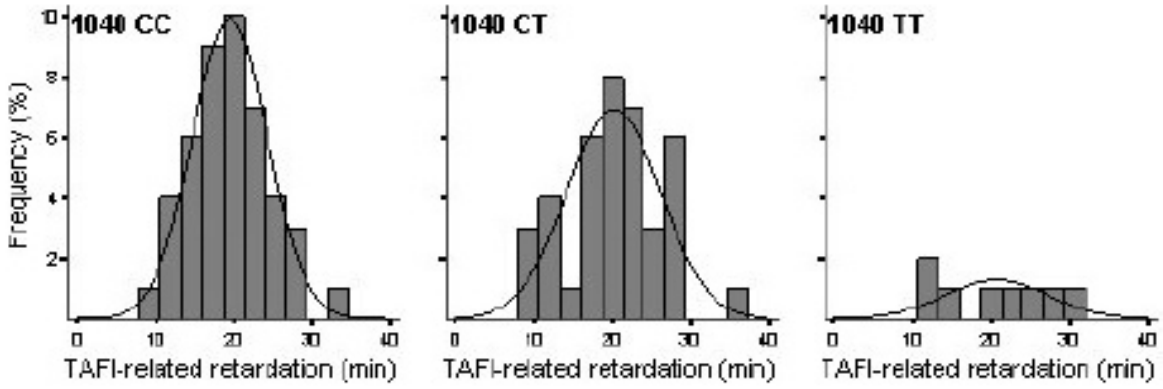

Figure 3. Frequency distribution of the TAFI-related retardation in the 1040 CT genotype subgroups of 92 healthy individuals. The TAFI-related retardation presented a normal distribution in each genotype subgroup.

times (LT) were derived for each sample in the absence and presence of PCI. Normal distributions were obtained for all assay parameters determined in plasma from 92 healthy individuals. Mean values for $LT_{-\ PCI}$ and $LT_{+\ PCI}$ and for TAFI-related retardation are presented in Table I. No associations were found between the $LT_{-\ PCI}$ or $LT_{+\ PCI}$ or the TAFI-related retardation and gender. There was also no relationship between TAFI-related retardation or the $LT_{-\ PCI}$ and age. The $LT_{+\ PCI}$, on the other hand, progressively decreased with age ($r = 0.311$; $P = 0.003$). In the genotype subgroups, normal distributions were observed for the $LT_{-\ PCI}$, $LT_{+\ PCI}$ (*not shown*) and the TAFI-related retardation (Fig.3, for 1040 CT genotype). The TAFI functional

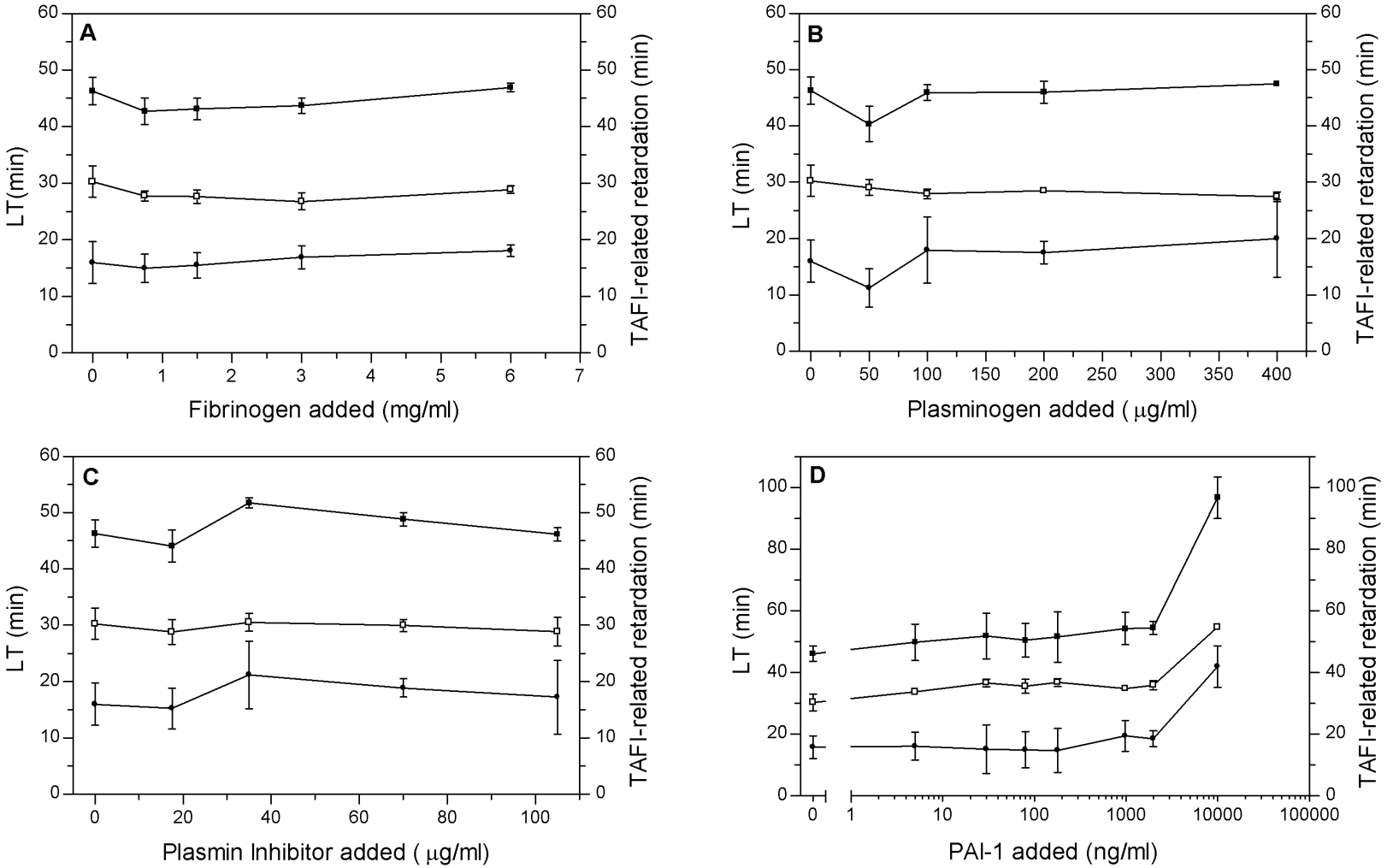

Figure 4. Influence of plasma components on the TAFI functional assay. Purified components were added in increasing concentrations to pooled normal plasma. Subsequently, the TAFI functional assay parameters were determined as described in the assay method. The lysis time (LT) in the absence (*closed squares*) and presence of PCI (*open squares*) and the TAFI-related retardation (*closed circles*) were plotted against the concentration of the component added. **A**, fibrinogen. **B**, plasminogen. **C**, plasmin inhibitor. **D**, PAI-1. Each point represents mean ± SD of at least 3 experiments.

Table I. Distribution of - 438 A/G and 1040 C/T SNPs of the TAFI gene in 92 healthy individuals and mean values of TAFI functional assay parameters, TAFI antigen levels and TAFI-specific antifibrinolytic activity.

	N (%)	LT_{-PCI} (SD)	LT_{+PCI} (SD)	TAFI-related retardation (SD)	TAFI antigen (SD) [a)]	TAFI-specific antifibrinolytic activity (SD)
- 438 A/G						
AA	7 (8)	52.6 (4.6)	33.3 (4.2)	19.5 (6.1)	0.94 (0.15)	21.4 (8.0)
AG	36 (39)	53.6 (5.7)	33.0 (3.6)	20.8 (5.9)	1.09 (0.17)	19.1 (4.6)
GG	49 (53)	51.8 (5.0)	32.7 (3.8)	19.3 (5.1)	1.21 (0.16)	16.0 (3.8)
1040 C/T						
TT (Ile/Ile)	8 (9)	53.5 (5.0)	32.9 (4.1)	20.7 (6.6)	0.98 (0.18)	21.6 (7.5)
CT (Thr/Ile)	39 (42)	53.2 (5.4)	33.3 (4.0)	20.2 (6.0)	1.10 (0.20)	18.4 (4.8)
CC (Thr/Thr)	45 (49)	51.9 (5.2)	32.5 (3.4)	19.5 (4.8)	1.21 (0.16)	16.2 (3.7)
Overall	92 (100)	52.6 (5.3)	33.0 (3.6)	19.8 (5.6)	1.14 (0.18)	17.6 (4.8)

TAFI functional assay parameters (LT_{-PCI}, LT_{+PCI}, and TAFI-related retardation) expressed in min. TAFI antigen levels were determined with the Electroimmunoassay and expressed in U/ml. The TAFI-specific antifibrinolytic activity was expressed in arbitrary units (AU).

[a)] TAFI antigen was significantly associated with TAFI genotypes, as previously described in Ref[24]. No association was found between the TAFI functional assay parameters and TAFI genotypes.

assay parameters were not associated with TAFI genotypes (- 438A/G and 1040C/T, tested by ANOVA).

Influence of other plasma components on the TAFI functional assay

We investigated the influence of a number of plasma components, namely fibrinogen, plasminogen, plasmin inhibitor and PAI-1, on the TAFI functional assay parameters. The assay was performed essentially as described under methods. Each of the purified components was added in increasing amounts to normal plasma which was then diluted 20-fold in TAFI-depleted plasma. As shown in Fig.4, fibrinogen (Fig.4A), plasminogen (Fig.4B) or plasmin inhibitor (Fig.4C) had no effect on the TAFI functional assay parameters (p > 0.05 for all, repeated measurements test) in a wide concentration range, up to a 3-fold increase of the component concentration in the normal plasma. An increase in PAI-1 concentration in normal plasma up to 2000

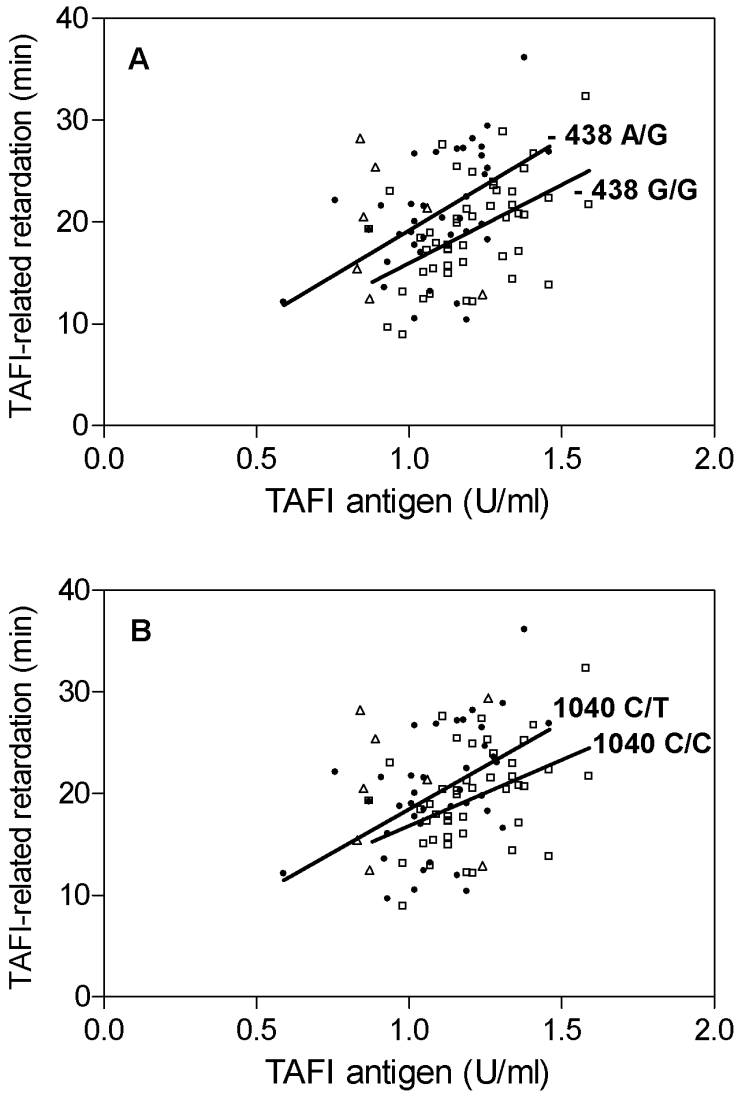

Figure 5. Effect of TAFI genotype on the relationship between the TAFI-related retardation and the TAFI antigen levels (Electroimmunoassay) for a group of 92 healthy individuals. **A**, -438 A/G genotype: (□) -438 G/G, (●) -438 A/G, (△) -438 A/A. **B**, 1040 C/T genotype: (□) 1040 C/C, (●) 1040 C/T, (△) 1040 T/T. Depicted lines were obtained by linear regression analysis of each of the genotype sub-groups.

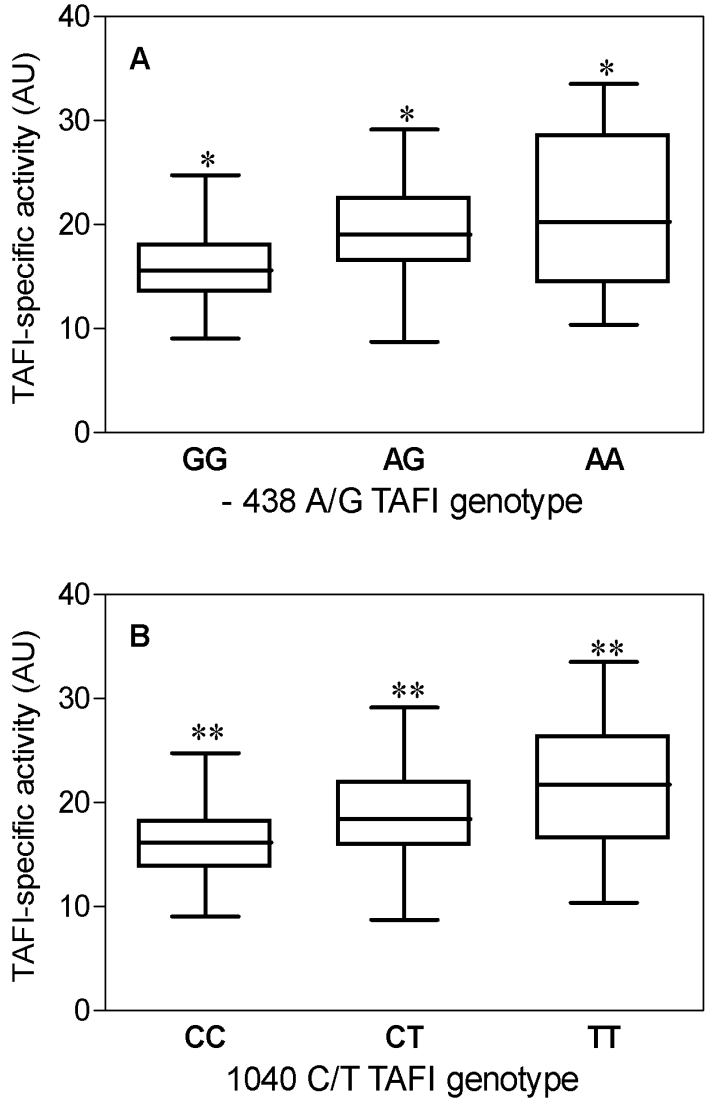

Figure 6. Box plots depicting the effect of - 438A/G and 1040C/T TAFI genotypes on the TAFI-specific antifibrinolytic activity in a group of 92 healthy individuals. **A**, - 438 A/G, *P = 0.004. **B**, 1040 C/T, **P = 0.003. The TAFI-specific antifibrinolytic activity was defined as the ratio between the TAFI-related retardation (min) and TAFI antigen level (U/ml) and was expressed in arbritary units (AU). P values for relations between TAFI-specific antifibrinolytic activity and TAFI genotypes were calculated by ANOVA – post-test for linear trend.

ng/ml (100-fold) had no effect on the TAFI functional assay parameters (Fig.4D - $p > 0.05$ for all, repeated measurements test). Only at PAI-1 concentrations of 10,000 ng/ml (500-fold increase) the TAFI functional assay parameters became considerably prolonged (96.6 ± 6.8 min against 46.1 ± 2.4 min for LT_{-PCI}, 54.7 ± 0.2 min against 30.3 ± 2.8 min for LT_{+PCI}, and 41.9 ± 6.8 min against 15.8 ± 3.7 min for TAFI-related retardation).
Correlation of TAFI functional assay parameters with TAFI antigen – Both the LT_{-PCI} and LT_{+PCI} displayed a weak correlation with the TAFI antigen levels (Table I) determined with the Electroimmunoassay (r = 0.205; P = 0.05 and r = - 0.245; P = 0.02, respectively). After correction for age, the association between LT_{+PCI} and the TAFI antigen levels became weaker. TAFI antigen levels were not associated with age or gender. The TAFI-related retardation correlated significantly with the TAFI antigen levels(r = 0.357; P = 0.0005).

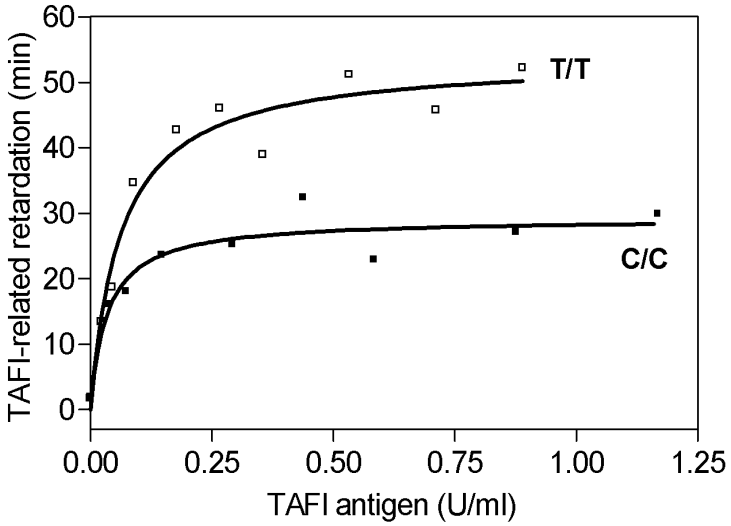

Figure 7. Dilution profile of a CC and a TT individual (1040C/C and 1040T/T, respectively) in the functional assay of TAFI. Variable TAFI concentrations were obtained by performing serial dilutions of individual plasmas in TAFI-depleted plasma. TAFI in each plasma sample was then activated during clotting with thrombin (3.3 NIH units/ml), thrombomodulin (0.6 units/ml) and $CaCl_2$ (20 mM) in the presence of r-tPA (0.10 µg/ml). Each sample was measured in the absence and in the presence of PCI and TAFI-related retardation was plotted against the TAFI antigen concentration. Each point represents the mean of at least two experiments. (■) CC individual plasma, (□) TT individual plasma.

When the different genotypes were taken into account the correlation of the TAFI-related retardation with the TAFI antigen increased in strength for the -438GG (r = 0.497, P = 0.0003), -438AG (r = 0.517, P = 0.0011) and the 1040CC (r = 0.430, P = 0.003), 1040 CT (r = 0.490, P = 0.0015) genotypes (Fig.5A and 5B). For the -438AA and 1040TT genotype subgroups no correlation was found between the TAFI-related retardation and the TAFI antigen levels, which may be due to the small sample sizes. The regression lines (Fig. 5B) are consistent with the notion that individuals with the 1040T allele (TAFI-325Ile) have higher TAFI activity (TAFI-related retardation) per TAFI antigen concentration than individuals with the 1040C allele (TAFI-325Thr).

Multivariate regression analysis illustrated that the TAFI antigen on its own explained 13% of the variation in the TAFI-related retardation and that the TAFI antigen together with the - 438AG and 1040CT genotypes clarified around 22% of the variation. Furthermore, the assay variability could explain roughly 50% of the variation in the TAFI-related retardation. This leaves nearly 30% of variability unexplained and points to additional differences between TAFI functional activity and TAFI level.

TAFI-specific antifibrinolytic activity

The TAFI-specific antifibrinolytic activity was defined as the ratio between the TAFI-related retardation (min) and the TAFI antigen level determined with the Electroimmunoassay (U/ml) and values are given in arbitrary units (AU) (Table I). The box plots in Fig.6 depict the association of the TAFI-specific antifibrinolytic activity with the - 438 A/G (Fig.6A) and 1040 C/T (Fig.6B) TAFI genotypes. From these results we concluded that individuals who are heterozygous or homozygous for the - 438A allele or for the 1040T allele have an increased TAFI-specific antifibrinolytic activity (by ANOVA, post-test for linear trend: r = 0.291; P = 0.004 and r = 0.306; P = 0.003, respectively). In addition, from the mean TAFI-specific antifibrinolytic activities in the TAFI genotype subgroups we can estimate the increase in antifibrinolytic activity due to the 1040 C/T TAFI SNP. On average, the subgroup with 325Thr/Ile (1040CT) and 325Ile/Ile (1040TT) showed respectively 14% and 34% greater specific antifibrinolytic activity than the subgroup with 325Thr/Thr (1040CC).

Dilution profile of TAFI variants

Dilution profiles were constructed with plasma from individuals with the 1040CC genotype and 1040TT genotype (Fig.7) in order to further examine the difference in TAFI-specific antifibrinolytic activity. Plasma of the CC individual displayed a response similar to that of the pooled normal plasma (Fig.2), in agreement with the high frequency of the C-allele in a normal population (Table I). Plasma of the 1040TT individual, on the other hand, exhibited a considerably higher response and plateau than that of the 1040CC individual, corroborating a higher specific antifibrinolytic activity of the 325Ile-isoform.

This distinct response means that pooled normal plasma cannot be used for the calibration of this TAFI functional assay.

Discussion

Thrombin activatable fibrinolysis inhibitor (TAFI) is a carboxypeptidase B zymogen present in plasma, which has the capacity to suppress fibrinolysis. This is accomplished by removing newly plasmin-generated C-terminal lysine and arginine residues in fibrin that function as high affinity binding sites for plasminogen [28,29], thus leading to the downregulation of plasmin generation [11,12,30].

To incorporate this role of TAFI in fibrin clot lysis into functional TAFI measurements, a novel assay was developed for the determination of TAFI functional activity in plasma. In order to mimic physiological conditions as much as possible, a high plasma concentration was used in the assay (80% plasma, final concentration). Fibrin generation and simultaneous TAFI activation were achieved by the addition of thrombin, thrombomodulin and calcium. The conditions for optimal TAFI activation, i.e. maximal retardation of clot lysis, have been described previously [9]. The lysis of the plasma clot is mediated by r-tPA that is included in the reaction mix and not in the plasma sample in order to minimise its inhibition by plasma proteinase inhibitors (such as PAI-1) prior to the assay measurements. Subsequently, the lysis profile is obtained by monitoring the OD at 405 nm while incubating the microtitre plate at 37°C. Finally, every sample is diluted 20-fold in TAFI-depleted plasma and determined in the absence and in the presence of potato carboxypeptidase inhibitor (PCI). The difference between clot lysis times in the absence and in the presence of PCI is thus specific to the contribution of TAFI. In order to study the assay performance we executed the TAFI functional assay in samples from 92 healthy individuals.

As shown in Fig.4, the 20-fold dilution in TAFI-depleted plasma ensures a minimal interference of other plasma components on the determination of TAFI functional activity in different individuals. In the particular case of PAI-1 (Fig.4D), it would be expected that when a 1:1 molar ratio between PAI-1 added and r-tPA used in the assay is reached an effect would be observed in the lysis times. To our surprise, at a PAI-1 concentration as high as 2000 ng/ml no effect was detected. The explanation possibly lies in the particular conditions of the TAFI functional assay. The presence of thrombin/thrombomodulin in the assay will activate protein C, which will form a tight 1:1 complex with PAI-1. The presence of an excess of thrombin may also lead to inactivation of PAI-1 via the formation of a ternary complex with vitronectin. Moreover, the assay is performed close to physiological conditions (pH 7.4 and 37°C), conditions under which PAI-1 is known to rapidly lose activity.

We also determined the TAFI antigen levels and genotyped the 92 healthy individuals for the - 438A/G and 1040C/T TAFI SNPs. Recently, it was shown that some anti-TAFI IgG preparations have different affinities towards the two TAFI isoforms (Thr325Ile) of the 1040C/T SNP [24,31]. This leads to a concentration- and genotype-dependent artefact in the TAFI antigen measurement. However, we have previously shown that some assays,

among which the TAFI Electroimmunoassay used in the present study, are insensitive to this artefact. By the use of such assays it was confirmed that the TAFI concentration in plasma is associated with the TAFI genotype, although TAFI antigen levels in 1040CC carriers were only 20 to 30% higher than in 1040 TT carriers [24].

A weak correlation was found between the TAFI-related retardation and TAFI antigen levels ($r = 0.357$). Taking into account the TAFI genotype improved the relationship between the TAFI-related retardation and TAFI antigen. The genotypes comprising the stable TAFI variant (TAFI-325Ile) displayed the highest response (upper line - Fig.5B). The dispersion found here between assays can only partially be accounted for by assay variability or by known TAFI variability (SNPs). This points to additional differences between TAFI molecules and/or their activity as well as to the need for more detailed information concerning the role of TAFI in distinct assays.

We studied the response of pooled normal plasma (Fig.2) as well as two genotype-specific individual plasmas (1040 CC and TT genotype, Fig.7). The genotype-specific plasma samples confirm a distinct response of samples with known differences in stability and activity with the more stable 325Ile-variant presenting a higher response and plateau than the 325Thr-variant. On the other hand, these results also indicate that pooled normal plasma cannot be used as the calibrator, as a combination of TAFI forms will generate an intermediate curve and will lead to under- or over-estimation of the individual samples.

Schneider *et al* [14] have shown that different isoforms of TAFI lead to different retardation profiles with distinct plateaux. Using purified recombinant variants they reported that the 325Ile-variant displayed a 30-50% higher antifibrinolytic effect, compared to the 325Thr-variant. We also found an increased TAFI-specific antifibrinolytic activity for the more stable 325Ile-variant (Fig.7) and accordingly, the specific TAFI antifibrinolytic activity was associated with the TAFI genotype (Fig.6). On average, the subgroup with 325Thr/Ile (1040C/T) and 325Ile/Ile (1040T/T) showed respectively 14% and 34% higher TAFI specific antifibrinolytic effect than the subgroup with 325Thr/Thr (1040C/C). This is consistent with the results of Schneider *et al* [14], especially when we take into account that here individual plasmas are being studied. The mechanism behind the distinct plateaux found by Schneider *et al* with recombinant TAFI variants and in this study with native TAFI variants from plasma remains unclear. During

the preparation of this manuscript Leurs *et al* [32] and Walker and Bajzar [33] demonstrated that TAFIa influenced clot lysis through a threshold-dependent mechanism. They showed that according to this mechanism not only the TAFI concentration, but also the time course of TAFI activation, the stability of TAFIa and the tPA concentration are determinant factors for the clot dissolution. This might help to elucidate the distinct retardation profiles observed in the present work (Fig.7 and in Ref.[14]) as the more stable variant will remain longer above the threshold.

It is interesting to note that the 325Ile-variant with the higher specific TAFI antifibrinolytic activity is present at a slightly lower concentration in plasma (Table I). This is explained by the linkage between the functional TAFI SNP (1040C/T) and SNPs in the TAFI promoter region. The two effects compensate for each other and, as a result, the TAFI functional activity (TAFI-related retardation) did not differ in the genotype subgroups (Table I). Accordingly, if TAFI functional activity is found to be associated with risk for some cardiovascular disease, it will be interesting to perform TAFI genotyping and TAFI antigen determination in order to investigate the contribution of these factors to the risk.

Thus, the TAFI functional parameter of this new assay (TAFI-related retardation) seems to be related to a particular combination of factors (e.g. concentration, stability, activity) for each individual. The understanding of these particular combinations and their relation to disease states will be necessary to further elucidate the involvement of TAFI in cardiovascular disease.

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CHAPTER 5

High functional levels of thrombin activatable fibrinolysis inhibitor (TAFI) are associated with an increased risk of first ischemic stroke

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Summary

Background and objective: Several studies have suggested that thrombin-activatable fibrinolysis inhibitor (TAFI) levels are associated with the risk of arterial thrombosis, but results have been contradictory. We studied functional TAFI levels and TAFI gene polymorphisms in 124 patients with a recent ischemic stroke and 125 age and sex-matched controls to establish the role of TAFI in ischemic stroke. *Methods and results:* Functional TAFI levels, defined as TAFI-related retardation, the difference in clot lysis time in the absence or presence of a specific activated TAFI inhibitor potato carboxypeptidase inhibitor (PCI), were higher in patients than controls (19.5 ± 4.2 vs. 17.7 ± 3.7 min, p <0.005). Clot lysis times in the presence of PCI, which were independent of TAFI, were also increased in ischemic stroke patients. This indicates that in these patients fibrinolysis is impaired not only by high TAFI levels, but also by other mechanisms. Individuals with functional TAFI levels in the highest quartile had an increased risk of ischemic stroke compared to the lowest quartile (OR 4.0, CI 1.6-9.8). In an unselected group of 36 of the 125 stroke patients functional TAFI levels were also measured at 3 months, and were persistently high. This indicates that increased functional TAFI levels after stroke are not caused by an acute phase reaction. No difference was found between patients and controls with respect to TAFI genotype distribution. *Conclusions:* Increased functional TAFI levels, resulting in decreased fibrinolysis, are associated with an increased risk of ischemic stroke.

Introduction

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a fibrinolysis inhibitor of which the role in the pathogenesis of ischemic stroke is not yet clear. The known physiological role of activated TAFI is the inhibition of fibrinolysis by removing C-terminal lysine and arginine residues of partially degraded fibrin, thereby slowing the rate of tissue plasminogen activator-induced plasminogen activation [1,2]. A role of TAFI in arterial thrombosis, including unstable angina pectoris and myocardial infarction, has been suggested. In patients with symptomatic coronary heart disease (CHD) contradictory results concerning TAFI antigen levels have been reported, some studies showed elevated levels in CHD [3-5], while others found decreased levels [6,7]. So far at least 14 polymorphisms in the TAFI gene have been identified, and some of these are strongly associated with plasma TAFI

antigen levels [8-10]. It has been suggested that TAFI gene polymorphisms may also be associated with the risk of acute myocardial infarction [7].

Studies on the role of TAFI in ischemic stroke are limited. One recent study found increased generated TAFI activity levels in patients with a history of ischemic stroke [11]. In addition, one other study in a small series of patients, showed increased TAFI antigen levels shortly after ischemic stroke [12]. Thus far, no studies have reported on the association between TAFI genotype and the risk of ischemic stroke. We studied functional TAFI levels and TAFI gene polymorphisms in patients with a recent acute ischemic stroke to further assess the possible association between TAFI and ischemic stroke.

Patients and Methods

Study design

We performed a case-control study with prospective inclusion of the participants. Cases were consecutively recruited patients with first ischemic stroke, admitted to the department of Neurology of our hospital. We used population controls, i.e neighbours or friends of the patients. They were age- and sex- matched, did not have a history of stroke and were not related to the patient.

Inclusion and exclusion criteria

Patients, controls and their parents had to be born in Northern Europe and be of the Caucasian race. Patients with a definite non-atherosclerotic cause of the stroke, such as a mechanical heart valve, endocarditis or dissection, as well as patients above 75 years or using oral anticoagulants were excluded.

Definitions and measurements

Ischemic stroke was defined as the acute onset of focal cerebral dysfunction due to cerebral ischemia with symptoms lasting more than 24 hours. Patients with TIA (symptoms lasting less than 24 hours) were included only if the neurological deficit was witnessed by a neurologist. In all patients a CT of the brain was made to confirm the diagnosis of ischemic stroke and to rule out hemorrhagic stroke. Clinical stroke subtypes were classified according to the OCSP (Oxford Community Stroke Project) criteria, modified by CT

[13]. Etiologic stroke type was classified according to the TOAST (Trial of Org 10172 in Acute Stroke Treatment) criteria [14]. We defined large artery atherosclerosis as a stenosis of presumed atherosclerotic origin in the symptomatic cervical artery of more than 50%, as determined by duplex ultrasonography and if necessary with angiography (NASCET method). Stroke severity was assessed with the Barthel index and the modified Rankin Scale [15]. Hypertension was defined as systolic blood pressure above 160 mm Hg or diastolic blood pressure above 90 mm Hg 5 days after the event, or the use of antihypertensive medication. Hypercholesterolemia was defined as fasting cholesterol level above 5.0 mmol/l or use of lipid lowering drugs. Diabetes mellitus was defined as the use of antidiabetic medication or glyco-Hb >6.5%.

In patients and controls detailed information about cardiovascular risk factors was collected. Patients were screened for cardiac abnormalities by means of standard twelve-lead ECG examination. A cardiologist was consulted in female patients aged 55 years or less, in male patients aged 45 years or less and in patients with ECG abnormalities or a history of cardiac disease. In these patients, 24 hours ECG monitoring and echocardiography was carried out. Screening for large vessel disease included duplex ultrasound or angiography of the carotid or vertebral arteries. Also information on medicaton was obtained: All patients used low dose aspirin (38 mg) at the time of first blood sample, 96 % of the patients at the time of second blood sample. The patients that were included were not treated with heparin, oral anticoagulants, clopidogrel, nor had they been previously treated with thrombolytic therapy.

Blood samples and procedures

Between 7-14 days after the stroke, venous blood samples were taken under strictly standardized conditions. The patients were in fasting condition, with no exposure to cigarette smoking or alcohol for at least the preceding 8 hours and blood was drawn after 15 minutes rest. Cholesterol levels and glyco-Hb were also measured 7-14 days after the ischemic stroke. In a random subgroup of 36 of the 124 stroke patients a second blood sample for functional TAFI and CRP measurement was collected when they visited our outpatient department three months after the stroke in the convalescence phase.

Blood was collected in citrate (0.105 M) using a Vacutainer system (Bekton Dickinson, Plymouth, UK.)

Blood was centrifuged (2000x*g* for 30 min at 4°C) and plasma was stored in aliquots at -80°C until use. Genomic DNA was isolated according to standard salting-out procedures.

Laboratory assays and TAFI genotyping

Recombinant tPA (Actilyse) was kindly supplied by Boehringer Ingelheim (Germany). Human thrombin and potato carboxypeptidase inhibitor (PCI), an inhibitor of TAFIa, were purchased from Sigma (St Louis, MO, USA) and Calbiochem (La Jolla, CA, USA), respectively. Rabbit lung thrombomodulin, with a specific activity towards thrombin of 1.2 units/µg, was acquired from American Diagnostica (Greenwich, CT, USA). TAFI-depleted plasma was prepared from a pool of normal plasma by using rabbit anti-TAFI antibodies immobilized with CNBr-activated Sepharose-4B. TAFI functional assay based on clot lysis was performed as described elsewhere [16]. Briefly, 100 µl of diluted plasma samples (20-fold dilution of individual plasmas in TAFI-depleted plasma) were added to the wells of a microtitre plate containing 25 µl of a reaction mix composed of thrombin, thrombomodulin, $CaCl_2$ and r-tPA (final concentrations in the assay: 3.3 NIH units/ml, 0.6 units/ml, 20 mM, and 0.10 µg/ml respectively), after which clots were formed. A control with addition of PCI was tested for each sample (final concentration 30 µg/ml). The wells were immediately covered with paraffin oil and the plate was placed in a pre-warmed incubation chamber (37°C, TECAN Sunrise® Microplate-reader). The optical density at 405 nm was monitored for 150 min. Lysis time (LT) was defined as the time point corresponding to a 50% decrease in optical density. Functional TAFI levels were calculated as TAFI-related retardation (RT), defined as the difference between the lysis time in the absence and in the presence of PCI, (LT_{-PCI} - LT_{+PCI}). The variation coefficients are 5.3% for LT_{-PCI} , 6.8% for - LT_{+PCI} and 12.9% for RT [16]. To exclude assay variation, plasma samples taken after 7-14 days were remeasured in the same run as the samples taken at three months.

Plasminogen activator inhibitor-1 (PAI-1) was measured using a commercially available ELISA (TintElize®PAI-1, Biopool, Umea, Sweden). Fibrinogen was measured according to the von Clauss method on a Sysmex automated coagulation analyzer (Dade Behring, Leusden, The Netherlands) (variation coefficient 3.5%). CRP was measured using a turbidometric assay obtained from Roche Diagnostics on a Hitachi 917 analyzer (Roche Diagnostics, Almere, The Netherlands) with a variation coefficient of 4%.

Determination of the TAFI -438A/G, 505A/G (Ala147Thr) and 1040 C/T

(Thr325Ile) genotype was performed using polymerase chain reaction (PCR) and subsequent restriction analysis, as previously described [6].

Statistics

For the TAFI 1040C/T (Thr325Ile) polymorphism with an expected prevalence of the TT genotype of 10% in the control group, the minimal detectable odds ratio (OR) associated with the TT genotype would be 2.8 (with an α=0.05, β=0.80). The relationship between the TAFI gene polymorphisms and ischemic stroke was calculated using logistic regression with adjustment for smoking, hypertension and diabetse and expressed as an OR with a 95% confidence interval (CI). The clot lysis times (LT) and TAFI-related retardation (RT) were divided into quartiles, based on the measurements in both cases and controls. The relationship between

Table 1. Baseline characteristics

	Patients (n=124)	Controls (n=125)
Demographics		
Age, yr (sd)	56 (±12)	56 (±12)
Female sex	58 (47%)	59 (47%)
Index event		
Stroke : TIA	115 : 9	NA
Stroke subtypes (TOAST criteria)		
Large vessel disease	11 (9%)	
Cardiac embolism	4 (3%)	
Small vessel disease	46 (37%)	
Undetermined etiology	61 (49%)	
Incomplete evaluation	2 (2%)	
Risk factors		
Smoking	61 (49%)	37 (30%)
Hypertension	60 (48%)	24 (19%)
Diabetes	18 (14%)	5 (4%)
Hypercholesterolemia	78 (63%)	84 (67%)
Oral contraceptives	11 (19%)	8 (14%)
Positive family history for cardiovascular disease	75 (61%)	56 (45%)
Previous DVT	6 (5%)	0 (0%)

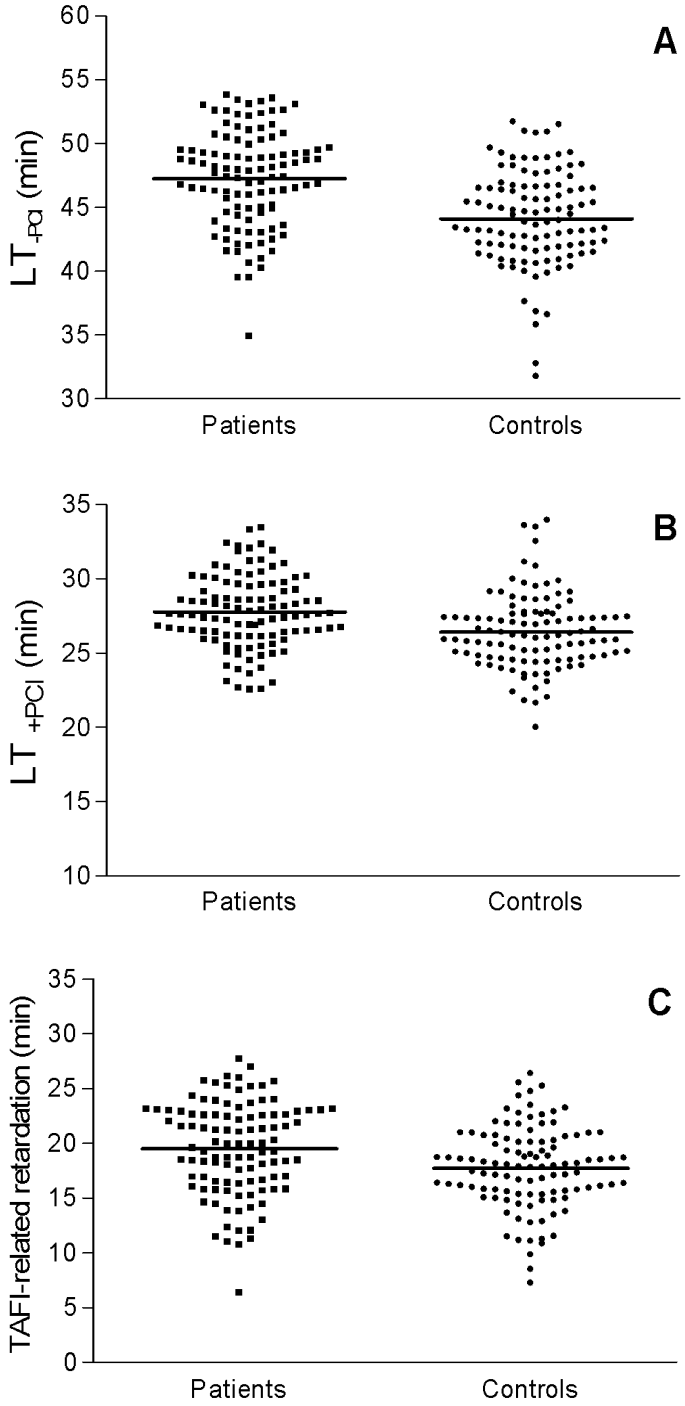

Figure 1. **A**, Clot lysis times (LT_{-PCI}); **B**, Clot lysis times (LT_{+PCI}) and **C**, functional TAFI levels (TAFI-related retardation, $RT=(LT_{-PCI}-LT_{+PCI})$ in patients with ischemic stroke and controls. The horizontal lines indicate mean value.

LT and RT and ischemic stroke was estimated by odds ratio per quartile relative to the quartile with the lowest levels. The association between TAFI haplotypes and the risk of stroke was investigated using (weighted) logistic regression with a modification of the method described by Tanck *et al* [17]. In addition to the effects of combined alleles, the effect of a single allele substitution was examined by pair-wise comparison of haplotype effects and this comparison was repeated for the different haplotypic backgrounds. Differences in functional TAFI levels among TAFI genotypes and among the

Table 2. Clot lysis times and functional TAFI in patients and controls

	Patients (n=106)	Controls (n=107)	P value*	Odds Ratio** (CI)
LT_{-PCI} (min)	47.3 ± 3.8	44.1± 3.7	<0.00001	1.25 (1.15-1.36)
LT_{+PCI} (min)	27.8 ± 2.5	26.4 ± 2.5	<0.0001	1.25 (1.11-1.41)
Functional TAFI (RT, min)	19.5 ± 4.2	17.7 ± 3.7	0.0012	1.12 (1.04-1.20)

Data are given as mean ± SD: LT_{-PCI} = clot lysis time in the absence of potato carboxypeptidase inhibitor (PCI); LT_{+PCI} = clot lysis time in the presence of PCI; RT = TAFI-related retardation ($LT_{-PCI} - LT_{+PCI}$); *two-sample t-test with equal variance. ** Odds ratio's are adjusted for smoking, hypertension and diabetes.

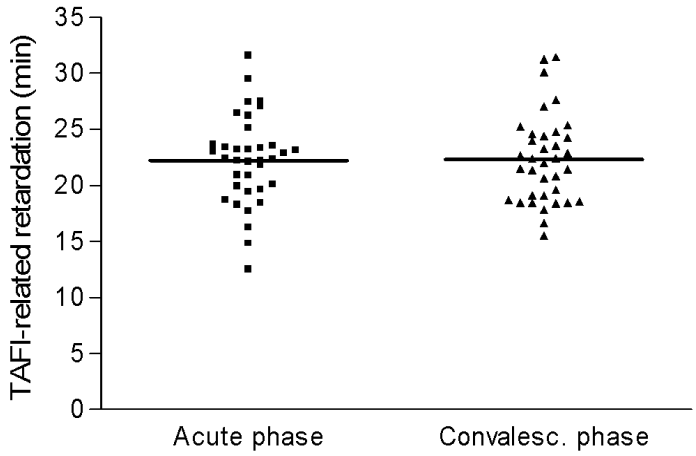

Figure 2. Functional TAFI levels (TAFI-related retardation, RT = (LT_{-PCI}-LT_{+PCI}) in patients with ischemic stroke at 7-14 days after ischemic stroke (acute phase) and after 3 months (convalescence phase). No significant difference was found (paired student's t-test, p =0.22).

groups were tested by one-way analysis of variance (ANOVA). The TAFI multilocus haplotype effects on functional TAFI levels were estimated using weighted linear regression [17]. The student's t-test was used to compare two groups and a p-value of <0.05 was considered significant.

Results

Patient population

The baseline characteristics of patients, including stroke subtypes, and controls are given in Table 1. Smoking, hypertension and diabetes occurred more frequently in the patients compared to controls. As expected, the risk of stroke (odds ratio, OR) for the classical risk factors in our study population were 2.3 (95% CI 1.4-3.9) for smoking, 3.9 (2.2-8.9) for hypertension, 4.1

(1.5-10.9) for diabetes, 0.8 (0.5-1.4) for hypercholesterolaemia, and 1.4 (0.6-3.6) for oral contraceptive use.

Functional TAFI levels in ischemic stroke

Functional TAFI levels were increased in ischemic stroke patients compared to controls (figure 1). Both clot lysis times (LT_{-PCI} and LT_{+PCI}) were significantly increased in patients with ischemic stroke compared to controls, which indicates less efficient fibrinolysis in patients with ischemic stroke compared to controls (figure 1, Table 2). We subdivided the individuals according to quartiles of functional TAFI levels. A significant association between functional TAFI levels and the risk of ischemic stroke was found. Individuals with the highest functional TAFI levels (highest quartile) had a markedly increased risk of ischemic stroke: OR of 4.0 (95% confidence interval (CI) 1.6-9.8) compared to the lowest quartile. Similar findings were obtained for LT_{-PCI} (OR 8.2, CI 3.2-21.3, highest quartile vs. lowest quartile).

Functional TAFI levels and the possible relation with acute phase response
The median CRP level was 5 mg/l (range 1-215 mg/l) in patients one week after the stroke and 3 mg/l (range 1-21 mg/l) in controls (p <0.05). After

Table 3. Association between clot lysis time (LT_{-PCI}) and TAFI-related retardation ($LT_{-PCI} - LT_{+PCI}$) and TAFI gene polymorphisms.

Genotype		Number		Clot lysis time (LT_{-PCI}) (min)		TAFI-related retardation (min)	
		P	C	Patients	Controls	Patients	Controls
TAFI 1040	CC	46	43	47.1 ± 4.2	43.6 ± 4.0	18.7 ± 4.3	16.9 ± 4.1
	CT	43	52	47.8 ± 3.3	44.6 ± 3.2	20.8 ± 4.1	18.3 ± 3.0
	TT	16	10	46.5 ± 3.8	43.2 ± 4.9	18.5 ± 4.0	16.9 ± 4.6
TAFI 505	GG	46	41	46.8 ± 4.0	43.4 ± 3.7	18.8 ± 4.2	17.1 ± 3.7
	AG	45	45	47.9 ± 3.6	44.0 ± 3.9	20.8 ± 4.3	17.9 ± 3.4
	AA	10	16	46.3 ± 4.0	46.0 ± 3.2	17.1 ± 3.9	18.3 ± 4.4
TAFI - 438	GG	51	49	47.4 ± 4.4	44.1 ± 3.9	19.2 ± 4.6	17.4 ± 4.2
	AG	41	46	47.4 ± 2.9	44.4 ± 3.1	20.1 ± 3.8	18.0 ± 2.9
	AA	14	9	46.5 ± 4.0	42.3 ± 5.4	18.8 ± 4.3	16.9 ± 4.8

Data given as mean ± SD

Table 4. Associations between TAFI genotype and risk of ischemic stroke.

	Genotype numbers andallele frequencies		
	Patients (n=124)	Controls (n=125)	**OR** (95% CI)
1040 C/T			
CC	55 (44%)	51 (42%)	reference
CT	51 (41%)	58 (48%)	0.82 (0.48-1.39)
TT	18 (15%)	12 (10%)	1.39 (0.62-3.13)
CT+TT	69 (56%)	70 (58%)	0.91 (0.55-1.51)
T	0.35	0.34	
505 G/A			
GG	54 (45%)	48 (41%)	reference
GA	53 (44%)	51 (44%)	0.93 (0.54-1.59)
AA	13 (11%)	18 (15%)	0.64 (0.29-1.43)
GA+AA	66 (55%)	69 (59%)	0.85 (0.51-1.42)
A	0.33	0.37	
- 438A/G			
GG	61 (49%)	61 (50%)	reference
GA	48 (38%)	50 (41%)	0.96 (0.56-1.63)
AA	16 (13%)	10 (8%)	1.60 (0.67-3.80)
GA+AA	64 (51%)	60 (49%)	1.07 (0.64-1.75)
A	0.32	0.29	

three months CRP values were decreased (median 2 mg/l, ranges 1-19mg/l) compared to the first blood sample, and were not significantly different from the controls. In our study population there was no correlation between CRP and functional TAFI levels (r= 0.06, n=199, *n.s.*). Plasminogen activator inhibitor-1 (PAI-1) antigen levels were not significantly different between cases (22.9 ± 13.5 ng/ml) and controls (25.1 ± 15.0 ng/ml) (p =0.23). A significant correlation was present between PAI-1 levels and functional TAFI levels (r= 0.18, p <0.01). There was no correlation between PAI-1 and LT_{+PCI} (r=0.02, *n.s*). A significant correlation was also present between functional TAFI levels and fibrinogen (r=0.19, p =0.01). Functional TAFI levels were also measured in the convalescence phase after three months in 36 patients. Functional TAFI levels were not significantly different between the acute and convalescence phase (RT was 22.25 ± 4.0 min vs. 22.33 ± 3.9 min, respectively, *n.s.*) (figure 2). Also LT_{+PCI} was still increased after 3 months 31.4 ± 0.3 min in patients and 26.4 ± 0.24 min for controls (p <0.0001).

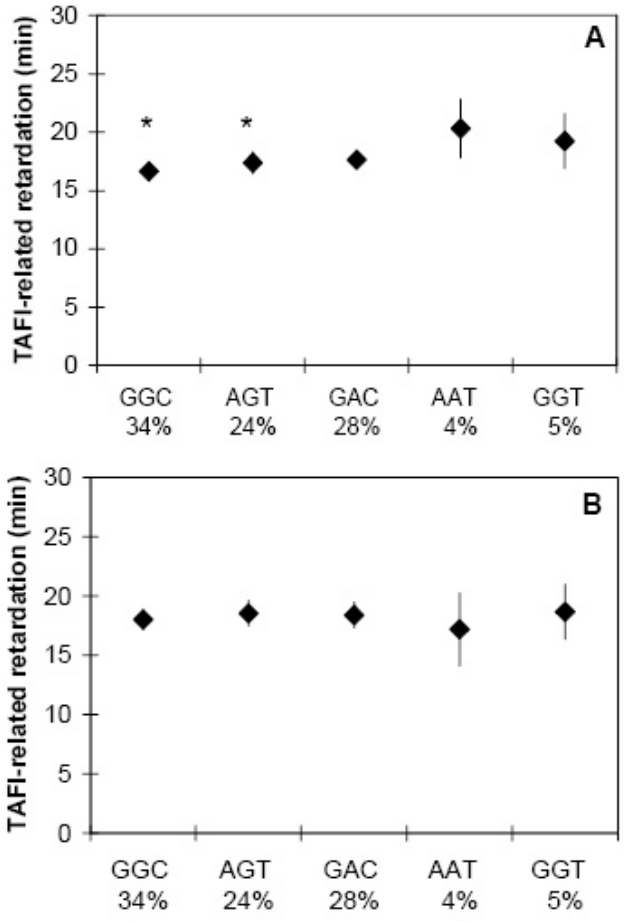

Figure 3. The effect of TAFI haplotypes on plasma functional TAFI levels (TAFI-related retardation) in controls (A) and patients with ischemic stroke (B) (levels are expressed as mean and 95% CI). The alleles in the haplotype are given in the following polymorphism order: -438G/A, 505G/A, and 1040C/T. Asterisks indicate haplotypes whose average effects are significantly different from the group with the highest functional TAFI level as the reference group (AAT). Haplotypes with a frequency below 2% were not included in the figure.

TAFI gene polymorphisms

Functional TAFI levels were not associated with the -438A/G, the 505A/G and the 1040C/T polymorphisms (Table 3). No association was observed between the three studied polymorphisms in the TAFI gene and the risk of ischemic stroke (Table 4). Also in subgroup analysis no association between TAFI polymorphisms and specific etiological subtypes of stroke, as mentioned in Table 1, was found (*not shown*).

Because there is a high degree of linkage disequilibrium between the three polymorphisms, it is not possible to estimate the effect of one single SNP on stroke risk by analyzing the single polymorphisms separately. Therefore haplotype analysis was performed to estimate more precise the contribution of a three individual polymorphisms on stroke risk. The effect of haplotypes on functional TAFI levels is shown in figure 3. The eight haplotypes did not differ in respect to functional TAFI levels in patients with ischemic stroke (Figure 3). In the controls the GGC and AGT haplotypes had significantly lower functional TAFI levels compared to the haplotype with the highest TAFI levels (AAT), which was taken as reference, although this was only a small difference (Figure 3). The risk of stroke was not significantly different

between the various TAFI haplotypes (*not shown*).

Association of functional TAFI levels and baseline characteristics

Functional TAFI levels were not different between males and females, individuals with and without diabetes and smokers and non-smokers. Individuals with hypertension had higher functional TAFI levels (RT 20.0 ± 3.8 vs 18.0 ± 4.1 min; p =0.0007). Also individuals with hypercholesterolemia (19.2 ± 3.7 vs 17.6 ± 4.5 min; p =0.0058) and of higher age (>45 years) (19.1 ± 4.0 vs 16.7 ± 4.0 min; p =0.0005) had increased functional TAFI levels. Both in young (<45 years) and elderly individuals functional TAFI was higher in stroke patients compared to controls (17.7 ± 4.2 vs. 15.4 ± 3.4 min, p = 0.06, and 20.0 ± 4.2 vs. 18.2 ± 3.6 min, p <0.005, respectively). The observed odds ratio's of clot lysis times (LT_{-PCI}) and functional TAFI levels did not change after adjustment for possible confounding variables including hypertension, diabetes, smoking, age and hypercholesterolemia (*not shown*). All other odds ratios given above are adjusted for these classical risk factors.

Discussion

In this study we found that increased functional levels of TAFI are associated with an increased risk of ischemic stroke. Clot lysis times in the presence of PCI, which were independent of TAFI, were also increased in ischemic stroke patients. This indicates that in these patients fibrinolysis is impaired not only by high functional TAFI levels, but also by different mechanisms.

Only two previous studies on TAFI levels in stroke patients have been reported so far. Montaner *et al* showed in a small study including 30 patients that TAFI antigen levels were increased in patients with ischemic stroke [12]. Plasma samples were taken within 24 hours of onset of the symptoms. Therefore the high TAFI antigen levels may be caused by an acute phase response, and may not reflect a causative role of TAFI in the development of ischemic stroke [3,18]. Santamaria *et al* performed a case-control study, comparable with our study design [11]. The patients were included at least one month after the event. They found that increased TAFI activity levels, measured using a synthetic substrate based assay, were associated with a 6-fold increased risk of ischemic stroke. The study population consisted of patients with at least one previous ischemic event, which may result in a selection of patients with recurrent thrombotic events, whereas in

our study only patients with a recent first episode of ischemic stroke were included. Despite these differences in patient population our study confirms the finding that increased functional TAFI levels are associated with an increased risk of ischemic stroke. By measuring functional TAFI levels at two different time points, and by including CRP in our analysis, we could establish that high functional TAFI levels are not due to an acute phase response.

The mechanism by which increased functional TAFI levels may result in ischemic stroke is still unknown. Hypothetically a decreased fibrinolytic potential will result in more stable thrombi, that are less prone to lysis. The relationship between PAI-1 and stroke supports such a mechanism and our findings on functional TAFI levels are in concordance with previous studies on the role of PAI-1 [19].

The role of TAFI in arterial thrombosis is still unresolved. Juhan-Vague *et al* showed that reduced TAFI antigen levels are found in patients with a history of acute myocardial infarction (AMI) [7]. We have previously shown in patients with unstable angina pectoris that lower TAFI antigen levels were found in patients who were refractory to medical treatment (more severe cases) compared to non-refractory patients [6]. On the contrary, other studies found increased levels of TAFI antigen in patients with arterial thrombosis, measured both in plasma of patients with symptomatic coronary heart disease or angina pectoris [3,5], or in blood obtained intracoronary during angiography [4]. A major difference between the various studies was the method of determination of TAFI antigen levels. It has become evident that some antigen assays have a variable antibody reactivity towards the different isoforms of TAFI [20,21]. The discrepancy between the studies may therefore (in part) be attributed to the various assays that have been used in the past. Because of the problems with TAFI antigen measurement, and the difficulties of interpretation of the various results, measurement of functional TAFI seems to be of utmost importance. In addition, genotype-independent TAFI antigen assays will be of value to establish the role of TAFI in arterial thrombotic disease.

There have been relatively few studies performed measuring TAFI activity levels in arterial thrombosis [11,22]. The tests used in previous studies were based on synthetic substrates. Several TAFI activity assays have recently been developed [23,24]. A clot lysis based assay is a more physiological method to study the impact of TAFI on fibrinolysis. However, a clot lysis–

based TAFI activity assay may be influenced by other plasma components [23,25]. The method that we have used is therefore very specific for TAFI activity. TAFI-related retardation (RT), calculated by the difference in clot lysis time in the absence and presence of a TAFIa inhibitor PCI, is a specific parameter for functional TAFI and is not influenced by other plasma components, including fibrinogen and PAI-1 [21]. The finding that TAFI-related retardation was significantly higher in patients compared to controls, indicates that functional TAFI levels are a risk factor for ischemic stroke. However, also clot lysis times in the presence of PCI are significantly higher in patients with ischemic stroke. This suggests that variables other than TAFI may also contribute to the increased risk of ischemic stroke. We adjusted for other potential confounders in our study population, such as hypercholesterolemia, age, and smoking, but these did not influence the association of clot lysis times with the risk of ischemic stroke. In the total study group, functional TAFI levels were higher in individuals with hypercholesterolemia, higher age and hypertension.

We found a weak, but significant correlation between PAI-1 antigen levels and functional TAFI ($r = 0.18$). However, PAI-1 antigen levels were not significantly different in patients and controls, and it is therefore unlikely that the increased risk of stroke associated with high functional TAFI levels is caused by a difference in PAI-1 [26]. In addition, *in vitro* experiments have shown that even extremely high levels of PAI-1 (up to 2,000 ng/ml) do not influence clot lysis times in our assay [16]. This indicates that PAI-1 levels will not influence the measurement of the functional TAFI levels in our stroke patients. A weak correlation was found between functional TAFI levels and fibrinogen ($r = 0.19$, $p < 0.05$). *In vitro* experiments however showed also that addition of high concentrations of fibrinogen did not alter clot lysis times and functional TAFI levels [16]. The weak correlations found between functional TAFI levels and both PAI-1 and fibrinogen, suggest a biological mechanism that influences functional TAFI levels, PAI-1 and fibrinogen in a similar way.

We studied three TAFI gene polymorphisms, one SNP in the promoter region and two SNP in the coding region. The -438 A/G was selected because it is a promoter gene polymorphism that is associated with TAFI antigen levels. The 505 A/G SNP was selected because it is also associated with TAFI antigen levels and with the risk of arterial thrombosis [7], and the 1040 C/T SNP was selected because this polymorphism encodes for two different isoforms of TAFI, with a difference in activity and half-life [10]. We

could not demonstrate a difference in the risk of ischemic stroke between the various genotypes. The -438 AA genotype seemed to be more frequent in patients compared to controls, however this is not significant, possibly because of the limited number of individuals in our study. Previous studies showed that the 505G/A SNP (Ala147Thr) in the TAFI gene is associated with the risk of acute myocardial infarction [7]. Individuals carrying the "TAFI antigen decreasing allele" (505G, Ala147) had an increased risk of acute myocardial infarction in a combined analysis of three case control studies. In our study we could not find this association between the TAFI 505G/A gene polymorphism and the risk of ischemic stroke. We could also not detect an association between the 1040 C/T polymorphism (Ile325Thr) and the risk of ischemic stroke. Because of the strong linkage between the three studied polymorphisms, haplotype analysis was performed to assess the effects of the individual polymorphisms, while excluding the effects of associated polymorphisms [17]. Only a small difference between functional TAFI levels in the different haplotypes was found (GGC and AGT versus AAT) in the controls. Since the AGT haplotype had significantly lower functional TAFI levels than AAT haplotype, it is suggested that the 505G (Ala147) allele may be associated with low functional TAFI levels. However, no significant difference was found between the GGC and the GAC haplotype. Further studies in larger cohort are required to establish these findings. No association was found between TAFI haplotypes and the risk of ischemic stroke.

One of the limitations of our study is the size of the study population, especially, as already mentioned above, for assessing the association between TAFI gene polymorphisms and ischemic stroke. It has therefore limited power to detect moderately high odds ratio's. In addition stroke is a more heterogeneous disorder than coronary heart disease. By using strict inclusion criteria for ischemic stroke patients, for instance excluding patients with a definite non-atherosclerotic cause for stroke, we cannot exclude the possibility that other specific etiological subtypes of stroke may be associated with TAFI genotypes. Based on the confidence intervals, however, TAFI gene variations will probably not be a strong risk factor for ischemic stroke.

Recently, it was reported that high TAFI antigen levels are associated with symptomatic hemorrhagic transformation in stroke patients treated with thrombolytic therapy (rt-PA) [27]. Patients with hemorrhagic transformation were found to have higher TAFI levels. This illustrates that TAFI may not

only be associated with risk, but also with outcome of ischemic stroke, albeit with a paradoxal mechanism [27].

We conclude that high functional TAFI levels are associated with an increased risk of ischemic stroke. The reduced fibrinolysis in stroke patients in our study, as shown by a prolongation of clot lysis times, can only partially be explained by TAFI. Other factors influencing fibrinolysis may be involved, for which additional studies should be performed. For future studies on TAFI it is important to study functional levels, antigen levels using genotype-independent assays, as well as genotype.

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THESIS

MODULATION OF THE PLASMINOGEN SYSTEM BY THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI)

MODULATION OF THE PLASMINOGEN SYSTEM BY TAFI

CHAPTER 6

Thrombin activatable fibrinolysis inhibitor (TAFI) affects fibrinolysis in a plasminogen activator concentration-dependent manner. Study of seven plasminogen activators in an internal clot lysis model.

Thrombosis and Haemostasis 2004; **91:** 473-79.

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Summary

TAFIa was shown to attenuate fibrinolysis. In our *in vitro* study, we investigated how the inhibitory effect of TAFIa depended on the type and concentration of the plasminogen activator (PA). We measured PA-mediated lysis times of plasma clots under conditions of maximal TAFI activation by thrombin-thrombomodulin in the absence and presence of potato carboxypeptidase inhibitor. Seven different PAs were compared comprising both tPA-related (tPA, TNK-tPA, DSPA), bacterial PA-related (staphylokinase and APSAC) and urokinase-related (tcu-PA and K2tu-PA) PAs. We derived the lysis time and the retardation factor which we plotted against the PA concentration. The retardation factor plots were bell-shaped. At low PA concentrations, the retardation factor was low, probably due to the limited stability of TAFIa. At intermediate PA concentrations the retardation factor was maximal (3-6 depending on the PA), with TNK-tPA, APSAC and DSPA exhibiting the strongest effect. At high PA concentrations, the retardation factor was again low, possibly due to inactivation of TAFIa by plasmin or to a complete conversion of glu-plasminogen into lys-plasminogen. Using individual plasmas with a reduced plasmin inhibitor activity (plasmin inhibitor Enschede) the bell-shaped curve of the retardation factor shifted towards lower tPA and DSPA concentrations, but did not decrease. In conclusion, TAFIa delays the lysis of plasma clots mediated by all the plasminogen activators tested. This delay is dependent on the type and concentration of the plasminogen activator, but not on the fibrin specificity of the plasminogen activator. Furthermore, plasmin inhibitor does not play a significant role in the inhibition of plasma clot lysis by TAFI.

Abbreviations

TAFI, thrombin activatable fibrinolysis inhibitor; TAFIa, activated thrombin activatable fibrinolysis inhibitor; TM, thrombomodulin; PCI, potato carboxypeptidase inhibitor; PA, plasminogen activator; tPA, tissue-type plasminogen activator; TNK-tPA, tenecteplase; DSPA, Desmodus rotundus salivary plasminogen activator; STA, staphylokinase; APSAC, anisoylated plasminogen-streptokinase activator complex; tcu-PA, two-chain urokinase-type plasminogen activator; K2tu-PA, amediplase; PI, plasmin inhibitor, previously α_2 –antiplasmin.

Introduction

Thrombin activatable fibrinolysis inhibitor (TAFI) is a zymogen present in plasma that can be activated (TAFIa) by thrombin or plasmin [1-4]. Thrombomodulin (TM) acts as a cofactor stimulating over 1000-fold the activation by thrombin [5-7]. Plasmin not only acts as an activator but can also inactivate TAFI and TAFIa by proteolytic cleavage at Arg^{302}, Lys^{327} or Arg^{330} [8]. TAFIa can cleave C-terminal lysine and arginine residues that become available when plasmin partially degrades fibrin [9-13]. These residues are binding sites for plasminogen and plasmin and essential for the upregulation of fibrinolysis as the activation of plasminogen is accelerated [14-16]and fibrin bound plasmin becomes more resistant to inactivation by plasmin inhibitor (PI), previously α_2-antiplasmin [17,18]. TAFIa blocks this upregulation and causes, possibly in this way, a prolongation of lysis times [9,10,12].

Given what is currently known about the mechanism of action of TAFI, we speculated that TAFIa would lead to variable inhibition of plasminogen activator (PA)-induced thrombolysis, depending not only on the concentration of the PA but also on the type of the PA. Different PAs operate through distinct mechanisms that rely to various extents on fibrin binding, on the C-terminal lysine-residues, on the conversion of glu- to lys-plasminogen and on the cofactor effect of soluble fibrin degradation products and of (DD)E [19-24].

Up to now, tissue-type PA (tPA) has been the PA of choice to study the effect of TAFI on fibrinolysis. TAFI-related retardation of tPA-mediated lysis has been described in purified systems [25], in clots prepared from diluted [10,12,26-28] and undiluted plasma [11], in whole blood clots [29] and in several animal models [9,30-32]. Walker *et al* [23] have recently studied the DSPA cofactor activities of TAFIa-treated soluble fibrin degradation products but still more insight is needed to understand the mode of action of TAFI with different plasminogen activators. Under our *in vitro* model conditions, we investigated how internal plasma clot lysis is affected by the PA concentration and how TAFIa changes the lytic activities of different PAs. Additional models, such as a model of external clot lysis, will be necessary to fully understand the multiple inter-linked mechanisms of TAFI and allow extrapolations to clinical practice of thrombolytic therapy.

A variety of plasminogen activators was tested in this study, from tPA-related plasminogen activators such as tPA itself, Tenecteplase (TNK-tPA)

and Desmodus rotundus salivary plasminogen activator (DSPA) to bacteria-related PAs such as staphylokinase (STA) and anisoylated plasminogen-streptokinase activator complex (APSAC), and urokinase-related PAs such as two-chain urokinase-type PA (tcu-PA) and Amediplase (K2tu-PA). The properties of these plasminogen activators have been described before [33,34]. To study the role of plasmin inhibitor in the TAFI-related retardation of lysis we used citrated plasma from a homozygous and a heterozygous individual for plasmin inhibitor Enschede [35,36]. This is a dysfunctional plasmin inhibitor molecule, which exhibits no inhibition of plasmin.

We demonstrated that TAFI delays clot lysis for all the PAs tested. No contribution of the plasmin inhibitor to the maximal TAFI-related retardation was found.

Materials and Methods

Materials

The tcu-PA (Ukidan) preparation was purchased from Serono. Staphylokinase (recombinant Sak42D) was a gift from Dr. H.R. Lijnen (University of Leuven). Other PAs were kindly supplied by the companies listed below: tPA (Actilyse), by Boehringer Ingelheim; TNK-tPA, by Genentech Inc; K2tu-PA (Amediplase), by Menarini; DSPA, by Schering AG; Anistreplase (Eminase), by Tramedico BV. Anistreplase concentration in mg/ml was calculated by using 1 IU/mg as specific activity. The tcu-PA concentration was determined spectrophotometrically using $A^{280}_{1\%,\ 1\ cm} = 13.6$ and its specific activity was 175,000 IU/mg. Human thrombin and potato carboxypeptidase inhibitor (PCI) were acquired from Sigma and Calbiochem, respectively. Rabbit lung TM, with a specific activity towards thrombin of 1.2 units/µg, was supplied by American Diagnostica Inc. Citrated platelet-poor plasma from ten healthy volunteers was obtained from the blood bank. A plasma pool was prepared and used in all normal plasma experiments. Citrated platelet-poor plasmin inhibitor (PI) deficient plasma from both a heterozygous and a homozygous individual with plasmin inhibitor Enschede was used when indicated.

TAFI Activation by Thrombin-TM Complex

Pooled blood bank plasma was dialysed against 50 mM Hepes, 100 mM NaCl, 20 mM tri-sodium citrate, pH 7.4 to remove phosphate ions and 100 µl were added to the wells of a microtitre plate containing 25 µl of a mix

per well. The mix was composed of thrombin (3.3 NIH units/ml), tPA (0.20 µg/ml), $CaCl_2$ (20 mM), PCI (0 or 30 µg/ml) and variable TM concentrations (0-1.8 units/ml) in a 50 mM Hepes buffer, pH 7.4 containing 0.1% w/v BSA. The concentrations between brackets refer to the final concentrations in the clotted plasma. The clots, prepared at room temperature, were immediately covered with 50 µl paraffin oil (Merck-107162) and the microtitre plate was placed in the incubation chamber pre-warmed at 37°C of a TECAN Sunrise Microplate-reader. The optical density was measured continuously at 405 nm. Lysis time is defined as the time point corresponding to a 50% decrease in optical density. The lysis time was determined by fitting lysis profiles with a sigmoidal regression equation.

TAFIa effect on PA-mediated clot lysis

Dialysed pooled blood bank plasma was added to the wells of a microtitre plate according to the procedure described above. A final concentration of 0.60 u/ml of TM was chosen in order to obtain maximal retardation of clot lysis under the assay conditions and the concentration of each of the seven PAs used was varied. The antifibrinolytic effect of TAFIa was described with the retardation factor, defined as lysis time in the absence of PCI divided by the lysis time in the presence of PCI. All the data result from the mean values of 3 independent measurements.

TAFIa effect on PA-mediated clot lysis in plasmin inhibitor Enschede plasma

Citrated plasmas from a heterozygous and a homozygous individual with PI Enschede were used in the procedure described above using both tPA and DSPA as plasminogen activators.

Plasmin inhibitor activity

Plasmin inhibitor activity was determined as described by Billing Clason *et al* [37] and expressed in % pooled plasma.

TAFI antigen concentration

A sandwich-type enzyme-linked immunosorbent assay (ELISA) was used to determined TAFI antigen levels in dialysed pooled blood bank plasma and in plasma of PI Enschede individuals. This ELISA made use of sheep polyclonal antibodies against TAFI (Affinity Biologicals Inc.). The levels were expressed in % pooled plasma, using as calibrator a citrated plasma pool composed of 40 normal individuals.

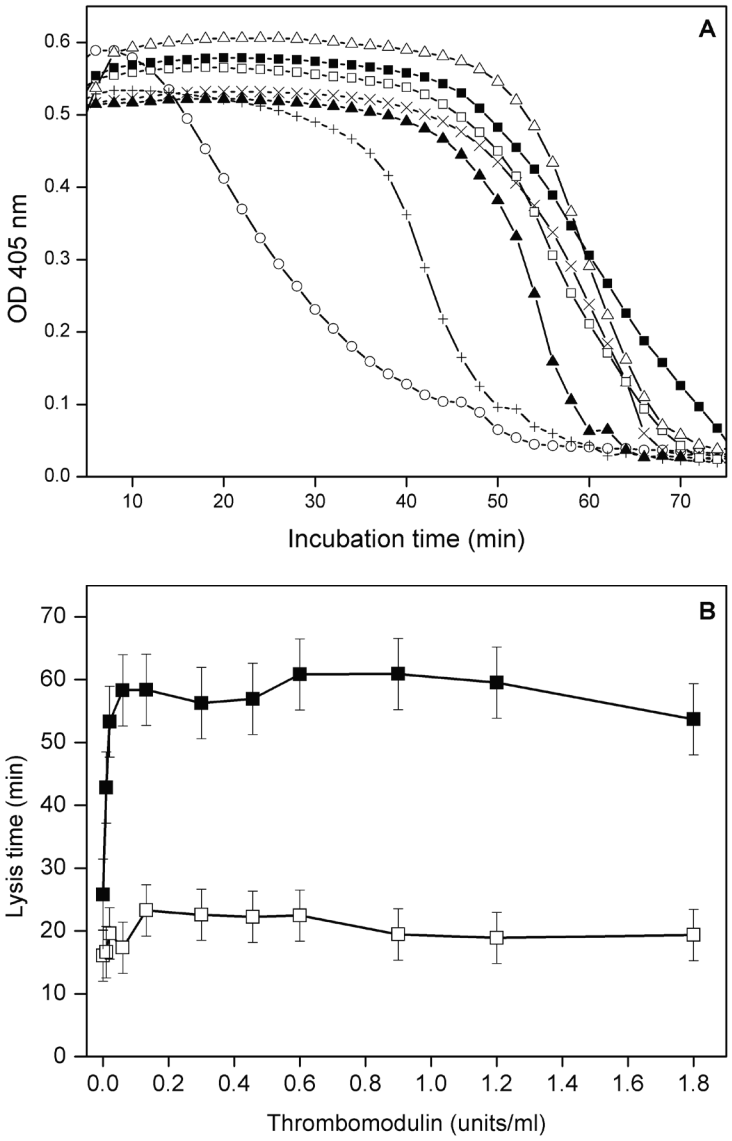

Figure 1.Thrombomodulin-induced delay of clot lysis by tPA (0.20 µg/ml). Citrated plasma with or without PCI (30 µg/ml) was clotted with 3.3 NIH units/ml thrombin and variable TM concentrations. **A**, A selection of clot lysis profiles in the absence of PCI: (○) No TM; (+) 0.01 unit/ml TM; (▲) 0.02 unit/ml TM; (X) 0.06 unit/ml TM; (□) 0.45 unit/ml TM; (■) 0.60 unit/ml TM; (Δ) 1.20 unit/ml TM. The baseline OD value was subtracted for each curve (about 0.37). **B**, Clot lysis times in the absence (■) and presence (□) of PCI. Lysis time was defined as the time point corresponding to a 50% decrease in OD 405 nm and the retardation factor as the lysis time in the absence of PCI divided by the lysis time in the presence of PCI. The error bars represent the SD.

Results

TAFI Activation by Thrombin-TM Complex

The tPA-mediated lysis of plasma clots prepared in the presence of variable TM concentrations is depicted in Fig.1A. Prolongation of lysis times by TM was dose-dependent in the absence of PCI. Even the lowest TM concentration tested (0.01 unit/ml) produced a considerable prolongation of lysis time. At a TM concentration of 0.06 unit/ml the maximum effect was achieved, resulting in a 3-fold prolongation of lysis time (Fig.1B). A small retardation of clot lysis by PCI was observed in the absence of TM that is probably due to TAFI activation by thrombin. In the presence of PCI there was no effect of the TM concentration on lysis time (Fig.1B). The prolongation of lysis

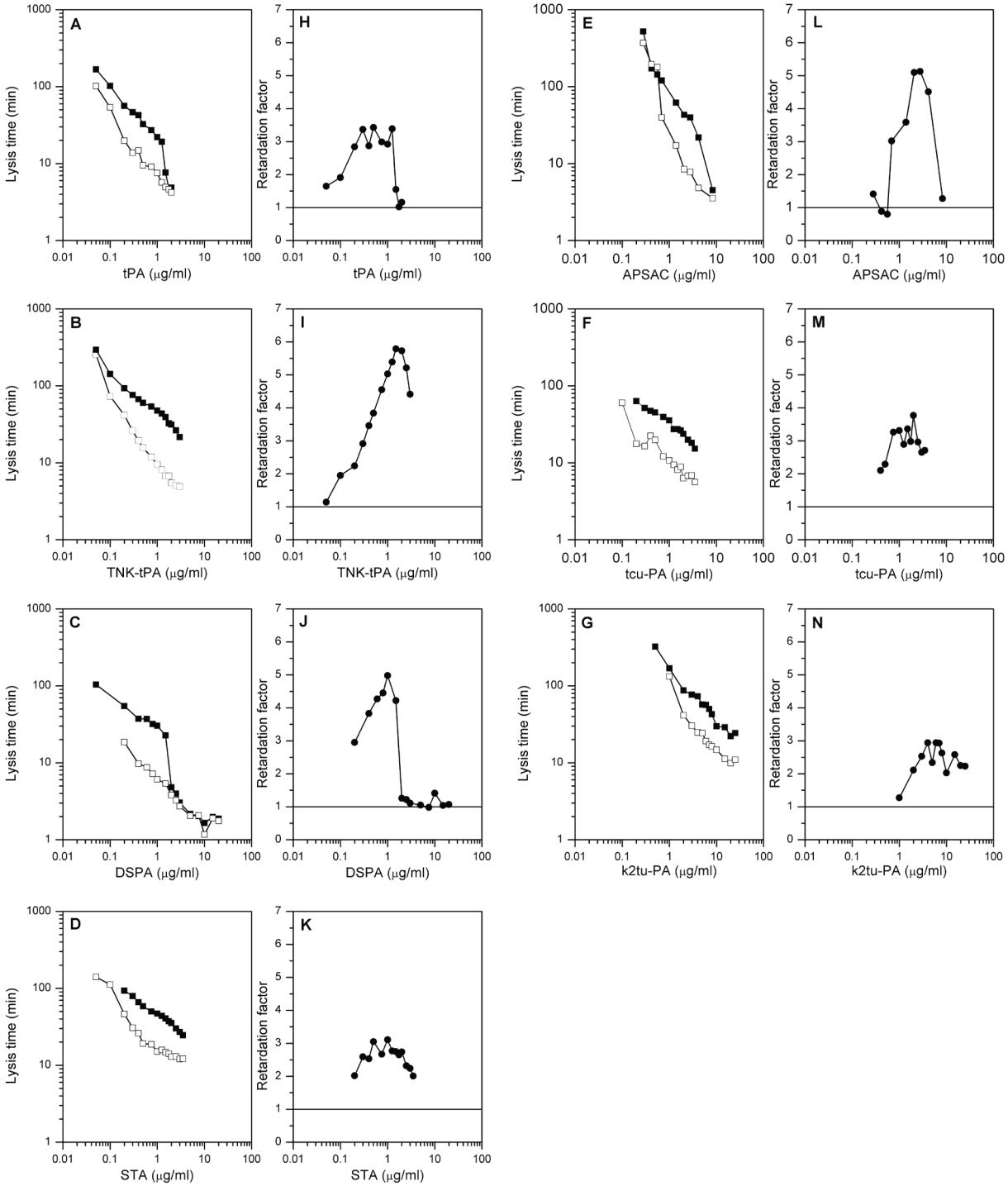

Figure 2. Effect of TAFIa on the internal lysis of plasma clots induced by seven PAs in the absence (■) and in the presence (□) of potato carboxypeptidase inhibitor (PCI). **A**, Tissue-type PA (tPA); **B**, Tenecteplase (TNK-tPA); **C**, Desmodus rotundus salivary PA (DSPA); **D**, Staphylokinase (STA); **E**, Anisoylated plasminogen-streptokinase activator complex (APSAC); **F**, Two-chain urokinase-type PA (tcu-PA); **G**, Amediplase (k2tu-PA). Retardation factor variations with PA concentration. **H**, tPA; **I**, TNK-tPA; **J**, DSPA; **K**, STA; **L**, APSAC; **M**, tcu-PA; **N**, k2tu-PA. Lysis time was defined as the time point corresponding to a 50% decrease in OD 405 nm and the retardation factor as the lysis time in the absence of PCI divided by the lysis time in the presence of PCI. (*line*) Retardation factor baseline.

time that could be quantitatively inhibited by PCI was attributed to TAFIa. The TAFI antigen concentration of the plasma pool was 96.4 ± 13.7%.

TAFIa effect on PA-induced lysis of plasma clots

Fig.2 shows the dependence of lysis times on the PA concentration in the internal clot lysis model with maximal TAFI activation. Seven different PAs were tested in the presence and absence of PCI (Fig.2A - G). For all the PAs, a decrease in lysis time with increasing PA concentrations was observed. The difference between the dose-response curves, in the absence and presence of PCI, depicts the potency of TAFIa antifibrinolytic activity, which was quantified through the retardation factor (Fig.2H - N). At low PA concentrations, long lysis times (100-500 min) were found and low retardation factors were derived. At intermediate PA concentrations the

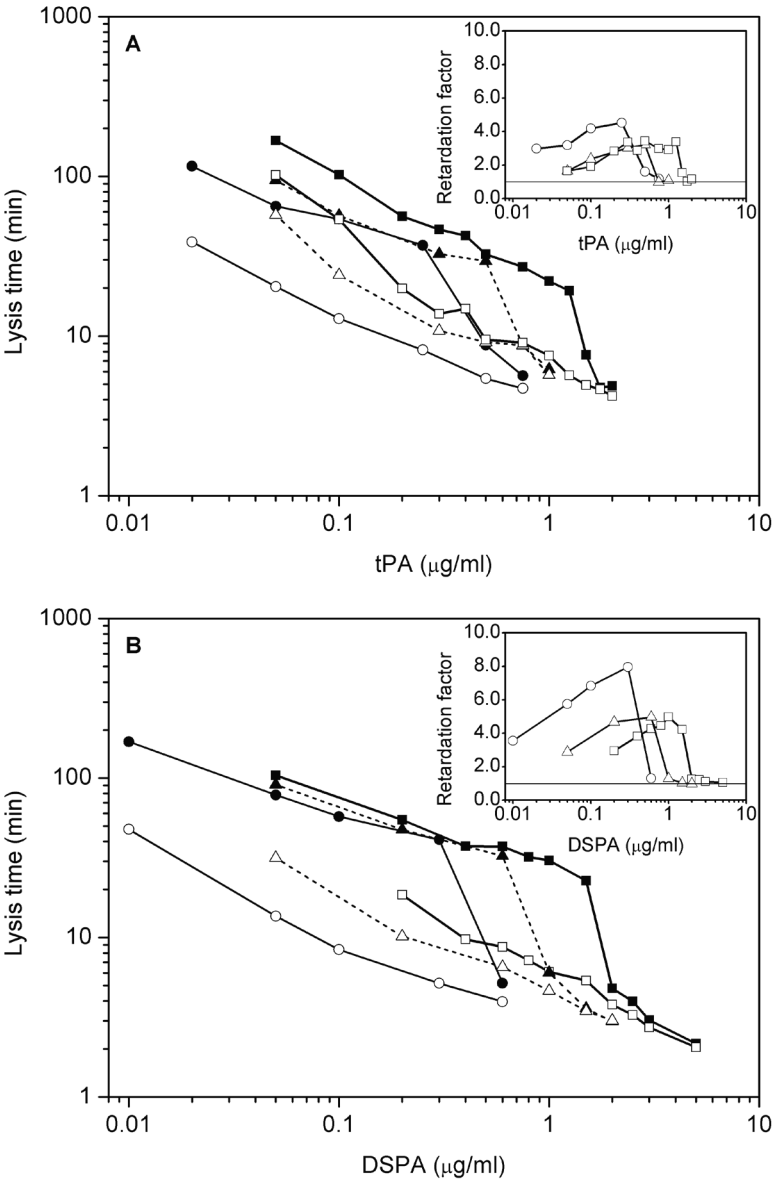

Figure 3. Effect of PI activity on the retardation of clot lysis by TAFIa. Normal plasma (*squares*), heterozygous PI Enschede plasma (*triangles*) and homozygous PI Enschede plasma (*circles*). Lysis of plasma clots induced by: A, t-PA and B, DSPA, in the absence (■,▲,●) and in the presence (□,Δ,○) of potatocarboxypeptidaseinhibitor (PCI). *Inset*, Retardation factor variation for normal plasma (□), heterozygous PI Enschede plasma (Δ) and homozygous PI Enschede plasma (○). Lysis time was defined as the time point corresponding to a 50% decrease in OD 405 nm and the retardation factor as the lysis time in the absence of PCI divided by the lysis time in the presence of PCI. (*line*) Retardation factor baseline.

retardation factor became maximal. The retardation factor declined again at high PA concentrations, in particular when short lysis times (<10 min) were obtained. All PAs generated bell-shaped retardation factor curves over variable concentration ranges. The maximal retardation factors were between 3-6. A powerful TAFI-related retardation (factor 5.8) was observed for TNK-tPA. The APSAC and DSPA-mediated plasma clot lysis was also considerably influenced by TAFIa (factor 5.1 and 5.0, respectively). tPA, the most common PA used to study the effects of TAFI on clot lysis, and tcu-PA showed an intermediate maximal TAFIa-dependent retardation factor (factor 3.4 for both). The weakest effect of TAFIa was observed for STA and K2tu-PA (factor 3.1 and 2.9, respectively).

The effect of TAFIa on PA-mediated clot lysis in plasmin inhibitor Enschede plasma

By using plasmin inhibitor Enschede plasma the role of plasmin inhibitor in the antifibrinolytic effect of TAFI was examined. Citrated plasma from both a heterozygous and a homozygous individual for PI Enschede was used. The PI functional activity was 7 ± 4 % for the homozygous individual and 51 ± 2 % for the heterozygous individual (% normal pool plasma). TAFI antigen levels did not differ significantly for the two individuals (73 ± 4 and 79 ± 9 %, respectively). Figure 3 shows that replacement of the pooled normal plasma by the PI Enschede heterozygous or homozygous individual plasma resulted in a shift towards shorter lysis times, both in the presence and in the absence of PCI and both for tPA (Fig.3A) and for DSPA (Fig.3B). Employment of the PI Enschede plasmas also resulted in a shift of the bell-shaped curves of the retardation factors towards lower tPA concentrations (inset, Fig.3A and 3B). The maximal retardation factor remained constant when pooled normal plasma was replaced by PI Enschede heterozygous plasma for both tPA and DSPA. When PI Enschede homozygous plasma was used the maximal retardation factor increased somewhat for both PAs tested (3 to 4.5 for tPA and 5 to 8 for DSPA). These results indicated that PI does not play a major role in the retardation of the clot lysis by TAFI.

Discussion

TAFIa, the activated form of thrombin activatable fibrinolysis inhibitor (TAFI), a plasma basic procarboxypeptidase, which is able to cleave C-terminal lysine and arginine residues from partially degraded fibrin [1-4]. These C-terminal lysine residues act as binding sites for plasminogen, and as fibrin is degraded by plasmin their number increases, enhancing plasminogen activation. TAFIa can delay lysis by preventing this enhancement of plasminogen activation. It has also been shown that the binding of plasmin to fibrin renders plasmin less susceptible to inactivation by plasmin inhibitor [17,18]. Yet, it is not known whether TAFI can delay lysis by preventing the protection of plasmin from inactivation by plasmin inhibitor.

Information concerning the effect of TAFI on clot lysis with different PAs is limited as most observations were done in systems with tPA-mediated lysis. Therefore, we performed this study to assess the antifibrinolytic potential of maximally activated TAFI in the lysis of plasma clots mediated by several types and concentrations of plasminogen activators. In addition, we were also interested in the role of plasmin inhibitor in TAFIa-related retardation of the internal lysis of a plasma clot.

Maximal TAFI activation was achieved by clotting plasma with thrombin and an optimal concentration of thrombomodulin in the presence of calcium ions (Fig.1) [11]. Seven different PAs (tPA, TNK-tPA, tcu-PA, APSAC, DSPA, K2tu-PA and STA) were tested with this model revealing that the presence of TAFIa prolonged lysis times for all PAs. The maximal retardation factor varied between 3- and 6-fold depending on the plasminogen activator. Moreover, we found that the effect of TAFIa was dependent on the PA concentration (bell-shaped curves in Fig.2H-N).

The effect was small at low PA concentrations (lysis times > 100 min), which we attributed to the restricted stability of TAFIa. Indeed, we have previously investigated the stability of TAFIa activity under similar conditions [11]. TAFIa activity declined rapidly, falling back to baseline levels within 60 min. Recently, low concentrations of PCI were shown to stabilize TAFIa, in particular at low tPA concentrations [27,38]. However, here we used a considerably higher PCI concentration which fully inhibits TAFIa. Altogether, this leads us to conclude that at low PA concentrations other fibrinolysis inhibitors play a more prominent role in our plasma clot lysis system than TAFI.

At high PA concentrations (lysis times < 10 min) the effect of TAFIa was

also low. We hypothesised that at these high PA concentrations elevated amounts of plasmin were generated. In our system, TAFI is activated by the thrombin/TM, which makes TAFIa available for proteolytic inactivation by plasmin [8]. Another possibility is that at high PA and high plasmin concentrations glu-plasminogen is fully converted into lys-plasminogen. Lys-plasminogen is produced by plasmin cleavage of the NH_2-terminal part of glu-plasminogen and presents a more open conformation than native glu-plasminogen. Moreover, the rate of activation of lys-plasminogen to plasmin is greater than that of glu-plasminogen. TAFIa seems to be specific to the glu-form of plasminogen [13]. The finding that in the absence of plasmin inhibitor activity and hence higher plasmin concentration the decrease in the effect of TAFIa occurred at lower tPA and DSPA concentrations corroborates the attribution of this decrease to a high plasmin activity (Fig.3).

The strongest TAFIa effects were observed for TNK-tPA and DSPA, two highly fibrin specific plasminogen activators. A moderate effect was, however, found for STA, which is also highly fibrin specific. Moreover, APSAC, which has low fibrin specificity, was amongst the PAs that displayed the strongest effect. This suggests that the magnitude of the inhibitory effect of TAFIa on internal plasma clot lysis does not depend strongly on the fibrin specificity of the plasminogen activator used.

Plasmin bound to C-terminal lysine residues is partially protected from inhibition by PI. TAFIa eliminates these binding sites and might therefore be able to modulate plasmin activity. Hence, TAFIa might rely on the rapid inhibition of unbound plasmin by PI to achieve its maximal lysis retardation effect. If this is the case, decreasing PI activity would lead to an increase in the plasmin concentration and to a faster lysis as well as a smaller TAFIa effect.

Keeping this in mind, we studied the role of PI in the mechanism of TAFIa retardation of clot lysis using plasmas from a heterozygous and a homozygous individual for PI Enschede. This dysfunctional PI molecule displays complete immunological identity with normal PI and has normal plasminogen-binding properties, but an abnormal functional activity resulting in a fully defective inhibition of plasmin [35,36].

The bell-shaped retardation factor curves shifted towards lower PA concentrations for both tPA and DSPA (Inset, Fig.3A and 3B, respectively). The maximal TAFIa-related retardation factor remained constant for the heterozygous PI Enschede individual and increased somewhat for the

homozygous individual. So, we can conclude that PI does not play a significant role in the inhibition of plasma clot lysis by TAFI.

The increase in maximal TAFIa-related retardation factor might be explained if this individual was also homozygous for the more stable and active variant of TAFI (325 Ile/Ile) [39]. Therefore, TAFI antigen for the PI Enschede individuals was determined by two immunological assays previously described by Gils *et al* [40] (*not shown*). The results showed that both the homozygous and the heterozygous PI Enschede individuals were not homozygous for Ile325. Another possible explanation would consist of TAFIa being able to remove the C-terminal lysine present in PI, interfering in this way with the rapid inactivation of plasmin [41]. However, recent work has demonstrated that Lys 436 in the plasmin inhibitor and not the C-terminal lysine residue (Lys 452) is necessary for the interaction with plasmin [42]. This implies that the increase in retardation factor remains still unexplained.

In conclusion, TAFI was found to affect the lysis of plasma clots mediated by all the PAs tested in broad concentration intervals. Also, the extent of the inhibitory effect of TAFI in the internal lysis of plasma clots was not determined by the fibrin specificity of the plasminogen activator. Moreover, the inhibitory effect of TAFIa on clot lysis did not depend on the presence of PI.

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CHAPTER 7

Fibrinolytic efficacy of Amediplase, Tenecteplase and scu-PA in different external plasma clot lysis models. Sensitivity to the inhibitory action of thrombin activatable fibrinolysis inhibitor (TAFI)

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Summary

In this study, the *in vitro* fibrinolytic efficacy of Tenecteplase, Amediplase and scu-PA was investigated in different external lysis models by measuring the lysis of human plasma clots after the addition of the plasminogen activators (PAs) to the surrounding plasma. The effect of TAFI was examined for each PA by neutralising TAFIa with potato carboxypeptidase inhibitor (PCI).

The lytic efficacy of Amediplase was lower than that of Tenecteplase at low PA concentrations but slightly higher at therapeutic concentrations. The activity of scu-PA was clearly lower than that of either Tenecteplase or Amediplase. The TAFI system inhibited external clot lysis mediated by all the PAs, when thrombomodulin was present in the model. In the therapeutic range (5-10 μg/ml) however, the TAFIa effect was negligible for both Amediplase and Tenecteplase. At lower PA concentrations the effect of TAFI on Amediplase was slightly stronger than that on Tenecteplase. Under static conditions the lysis rates were lower than with stirring. The role of TAFI was similar under both conditions.

In conclusion, at therapeutic concentrations Amediplase was slightly more active than Tenecteplase and scu-PA under all conditions used. Therefore Amediplase might possibly be a more potent thrombolytic agent at these concentrations and increase the efficacy of thrombolysis. The potential of TAFI for inhibiting thrombolytic therapy is probably low. However in conditions where the local PA concentrations are sub-optimal TAFI might affect the lysis rate.

Abbreviations

TAFI, thrombin activatable fibrinolysis inhibitor; TAFIa, activated thrombin activatable fibrinolysis inhibitor; TM, thrombomodulin; PCI, potato carboxypeptidase inhibitor; PA, plasminogen activator; tPA, tissue-type plasminogen activator or Alteplase; TNK-tPA, Tenecteplase (Metalyse); K2tu-PA, Amediplase; scu-PA, single-chain urokinase-type plasminogen activator or pro-urokinase.

Introduction

Plasminogen activators (PAs) comprise the serine proteinases, which are responsible for the conversion of plasminogen, the inactive precursor circulating in blood, into plasmin required for the proteolytic degradation of fibrin (1). The elucidation of the molecular mechanisms of physiological fibrinolysis has led to the development of new thrombolytic agents (engineered PAs) that constitute the current standards for thrombolytic therapy.

Tenecteplase is a genetically engineered tissue-type plasminogen activator (t-PA or Alteplase) variant with a few substitutions in the amino acid sequence, which exhibits a prolonged half-life in the circulation and a decreased sensitivity to plasminogen activator inhibitor-1 (2) while maintaining affinity to fibrin (3). Amediplase (K2tu-PA) is a single-chain hybrid PA, comprising the kringle 2 of t-PA and the protease domain of single-chain urokinase-type plasminogen activator (scu-PA or pro-urokinase) (4). t-PA has a strong affinity to fibrin (5), which is ascribed to the finger domain and to a lesser extent to its kringle 2 domain (6,7) whereas scu-PA has hardly any affinity for fibrin (8). Due to the presence of kringle 2 of t-PA, Amediplase has a moderate affinity to fibrin (4,9). scu-PA can be inactivated by direct cleavage of the protein chain by thrombin (10). Moreover, this reaction is accelerated by thrombomodulin (11). Amediplase, on the other hand, does not contain the thrombin-sensitive site of scu-PA and is probably not directly inactivated by thrombin. Tenecteplase and Alteplase are nowadays widely used for thrombolytic therapy, while Amediplase is currently undergoing a phase III clinical trial in patients with myocardial infarction (12).

In the present study, we compared the lytic efficacy of three distinct PAs (Tenecteplase, Amediplase and scu-PA) in external plasma clot lysis, which is a model for thrombolytic therapy. In such a model, the PA is applied outside the plasma clot and proteolytic degradation occurs in a sharp active zone on the exterior edge of the clot (13). This active zone reflects the accumulation of plasminogen and PA from the plasma clot exterior (14). The accumulation of PA varies for different PAs according to their affinity for fibrin.

Thrombin activatable fibrinolysis inhibitor (TAFI) is an inhibitor of the fibrinolytic system (15) although its part in thrombus resistance to thrombolytic therapy is still rather unclear. TAFI is a plasma procarboxypeptidase B, which is activated to TAFIa by thrombin. Thrombomodulin works as

a cofactor for this reaction resulting in a 1000-fold acceleration of TAFI activation (16). TAFIa then eliminates carboxyterminal lysine residues from partially degraded fibrin, thereby inhibiting the binding of plasminogen to partially degraded fibrin as well as the lysis rate (17). Recently, we described an internal plasma clot lysis model where the PA is incorporated into the plasma clot during clot formation and demonstrated that all PAs tested are sensitive to the inhibitory action of TAFIa (18). Maximal TAFI retardation of the clot lysis occurred at intermediate PA concentrations and varied between 3- and 6-fold. At low PA concentrations lysis times were long and the inhibitory effect of TAFIa was low probably due to the limited stability of TAFIa. At high PA concentrations, the inhibitory effect of TAFIa was low and possibly related to the inactivation of TAFI/TAFIa by the elevated amounts of plasmin generated under these conditions.

The sensitivity of Tenecteplase, Amediplase and scu-PA to the inhibitory action of TAFIa was investigated in a modified external plasma clot lysis model, which is sensitive to TAFI. Variable stirring conditions were applied, striving to accomplish variable transport rates of the fibrinolytic proteins.

Materials and Methods

Materials

Amediplase (batch P003/03) was supplied by Menarini Biotech (Rome, Italy). Tenecteplase (Metalyse) was from Boehringer Ingelheim (Ingelheim, Germany). scu-PA from a transformed human kidney cell line was provided by Sandoz (Vienna, Austria) and biochemically characterised previously (19). Potato carboxypeptidase inhibitor (PCI) was acquired from Calbiochem (EMD Biosciences, Merck, Darmstadt, Germany). Rabbit lung thrombomodulin (TM), with a specific activity towards thrombin of 1.2 units/µg, was supplied by American Diagnostica Inc (Greenwich, CT, USA). Batroxobin maranhao was from Pentapharm (Kordia, Leiden, the Netherlands). Recombinant hirudin (Refludan/Lepirudin) and human thrombin were purchased from Aventis Behring (ZLB Behring, King of Prussia, PA, USA) and Sigma (St Louis, MO, USA), respectively. Trasylol (aprotinin) was obtained from Bayer (Leverkusen, Germany). Human fibrinogen (plasminogen, von Willebrand factor and fibronectin depleted) was supplied by Enzyme Research Laboratories (Kordia BV, Leiden, the Netherlands) and labelled with fluorescein isothiocyanate (FITC) (Sigma)

as described elsewhere (14). A plasma pool was prepared with citrated apheresis plasma from 12 healthy donors from the local blood bank.

Defibrinated plasma

Plasma was dispensed into plastic tubes containing 2.5 BU/ml batroxobin, final concentration. After incubation for 2 hours at room temperature, the clots were disconnected from the walls of the tubes and centrifuged at 5000g for 30 min. The resulting plasma supernatant was pooled into a tube and subsequently subdivided into 1 ml aliquots, which were kept at -80°C. Batroxobin is a thrombin-like proteolytic enzyme isolated from snake venom which splits the 16 Arg-17 Gly bond in Aα-chain of fibrinogen. Batroxobin only causes release of fibrinopeptide A and no TAFI activation occurs during this plasma preparation [20].

External plasma clot lysis model

External plasma clot lysis was measured essentially as previously described (9) with some modifications. Plasma was supplemented with FITC-labelled fibrinogen and aliquots of 0.2 ml were clotted with calcium chloride (20 mM) and thrombin (1 NIH U/ml) for 30 min at 37ºC. The clots were disconnected from the tube wall and immersed in 1.0 ml of plasma supplemented with hirudin (0 or 20 ATU/ml) and with Amediplase, Tenecteplase or scu-PA (0-10 µg/ml). Concentrations between brackets refer to final concentrations. The clots were incubated for 6 hours at 37ºC on a shaker. At regular intervals samples were taken from the plasma and diluted in 50 mM Hepes buffer, pH 7.4 containing 0.1% w/v BSA. The released FITC-labelled fibrin degradation products were immediately determined in a spectrofluorometer and the extent of lysis calculated for each time point. The signal of the FITC-labelled fibrinogen supplemented to plasma was considered as 100%. The extent of lysis was plotted against the sampling time and the half-maximal lysis time was determined by fitting these lysis profiles with a sigmoidal regression equation.

External plasma clot lysis model sensitive to TAFI

Plasma (125 µl) was supplemented with FITC-labelled fibrinogen and clotted by the addition of calcium chloride (20 mM) and thrombin (0.3 NIH units/ml) for 30 min at room temperature. Then the plasma clots were disconnected from the wall and immersed in defibrinated plasma (125 µl) supplemented with calcium chloride (20 mM), thrombomodulin (0.1 units/ml) and Amediplase, Tenecteplase or scu-PA (0-10 µg/ml). Concentrations

between brackets refer to final concentrations. Control experiments with potato carboxypeptidase inhibitor (PCI, 30 µg/ml), a specific inhibitor of TAFIa, both incorporated in the clot and added to the surrounding plasma, were performed in parallel. The incubation was performed at room temperature with or without a shaker to create different stirring conditions. Sample collection and calculations were performed as for the external plasma clot lysis model described above. Values for extent of lysis represent the mean of six repeats. An unpaired t-test was used to test for differences between half-maximal lysis times of distinct PAs at specific concentrations. P values < 0.05 were considered statistically significant.

Fibrin content

The external plasma clot lysis model sensitive to TAFI was used to reinforce the results obtained in the presence of PCI. Plasma clots were prepared without addition of FITC-labelled fibrinogen and external lysis was induced as described above using 5 µg/ml of either Tenecteplase or Amediplase. After 30 min plasma clot lysis was stopped by transferring the remaining plasma clot into a well containing 250 µl of Trasylol (100 units/ml) in 50 mM Hepes, pH 7.4. The clot was incubated on an orbital shaker for 60 min, time after which the Trasylol solution was refreshed. During the next 24 hours, the clots were thoroughly washed with 50 mM Hepes, 1.5 M NaCl, pH 7.4 (refreshed 3 times) and afterwards with 50 mM Hepes, pH 7.4 (refreshed 3 times). The buffer was then removed and the clots squeezed to minimize buffer content. The clot was dissolved in 200 µl 2.5 M NaOH (80^{o}C, 4 hours) and the protein content (21) estimated with sodium carbonate and Folin-Ciocalteu's phenol reagent. The fibrin content of plasma clots treated in the same way but not subjected to external lysis was considered as 100% (mean, n = 6). The variation coefficient for these clots was 4.8 %. An unpaired t-test was used to compare the remaining fibrin content in the plasma clot after external lysis mediated by either Tenecteplase or Amediplase. P values < 0.05 were considered statistically significant.

Results

External plasma clot lysis model

Fig.1 shows the time-dependent results of external plasma clot lysis, in which the PAs were added to the external plasma in increasing concentrations (0-10 µg/ml).

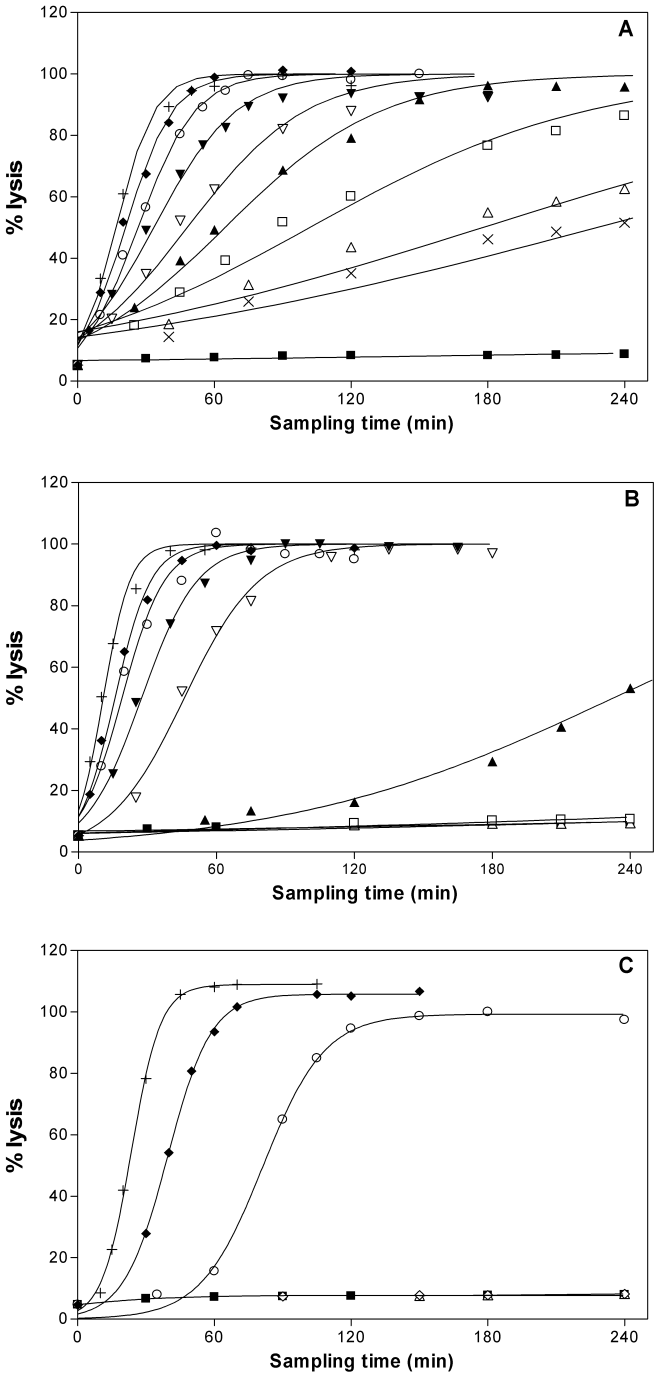

Figure 1. Profiles for the extent of external plasma clot lysis mediated by: **A**, Tenecteplase; **B**, Amediplase; **C**, scu-PA. Preformed plasma clots labelled with FITC-fibrin were immersed in citrated plasma containing the thrombin inhibitor hirudin (20 ATU/ml) and increasing amounts of the plasminogen activators: (■) 0 µg/ml; (×) 0.031 µg/ml; (Δ) 0.078 µg/ml; (□) 0.156 µg/ml; (▼) 0.31 µg/ml; (▽) 0.63 µg/ml; (Δ) 1.25 µg/ml; (○) 2.5 µg/ml; (◆) 5 µg/ml; (+) 10 µg/ml. The time-dependent release of FITC-labelled fibrin degradation products into the external plasma was determined with a fluorometer, expressed as a percentage of lysis and plotted against the sampling time.

The PAs showed sigmoidal lysis curves. The half-maximal lysis times were derived for each lysis profile and presented in Fig.2 as a function of the PA concentration. Amediplase was less active (i.e. longer half-max lysis times) than Tenecteplase at low PAs concentrations, but slightly more active than Tenecteplase at high, therapeutic concentrations. scu-PA (pro-urokinase) was less active than both Amediplase and Tenecteplase. Moreover, lysis experiments were performed with (Fig.1 and Fig.2) and without (not shown) the thrombin inhibitor hirudin (Refludan 20 ATU/ml). The purpose of this was to study the effect of thrombin generation/activity and TAFI activity,

but no significant differences were observed in the absence or presence of hirudin. This indicates that the amounts of thrombin in the system were too low for substantial TAFI activation. In addition, this indicated that the direct inactivation of scu-PA by thrombin was also minor.

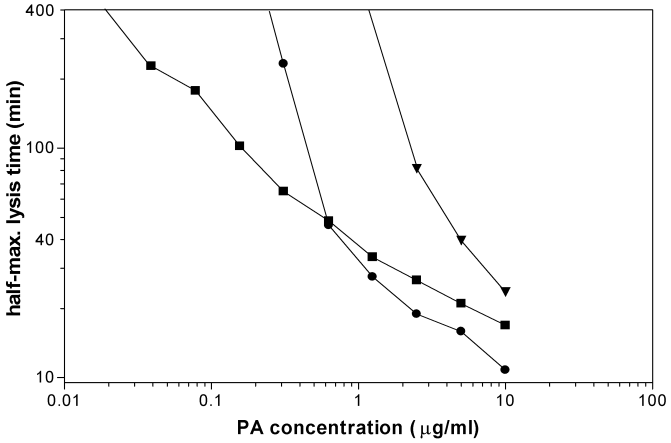

Figure 2. Comparison of the half-maximal lysis times obtained for Tenecteplase (squares), Amediplase (circles) and scu-PA (triangles) in the presence of hirudin (20 ATU/ml).

External plasma clot lysis model sensitive to TAFI

To be able to compare the sensitivity of the different PAs towards the inhibitory action of TAFI the external plasma clot lysis set-up was modified, as described under methods. To enhance TAFI activation and to increase the half-life of TAFIa, thrombomodulin was added to the external defibrinated plasma and experiments were performed at room temperature, respectively. Stirring increased the extent of plasma clot lysis both in the absence and in the presence of PCI and shifted the dose-response curves at each time point towards lower PA concentrations. Inclusion of PCI resulted in a clear acceleration of external plasma clot lysis for each of the three PAs.

The time-dependent lysis profiles were constructed and used to determine the half-maximal lysis time, which was then plotted against the PA concentration both with (Fig.3) and without (*not shown*) stirring. At low PA concentrations, long half-maximal lysis times were observed as well as the high inhibitory effects of TAFIa. These effects were more pronounced for Amediplase and scu-PA than for Tenecteplase. The inhibitory effect of TAFIa decreased with increasing PA concentrations. In the therapeutic range (5-10 μg/ml), the TAFIa effect was negligible for both Amediplase and Tenecteplase.

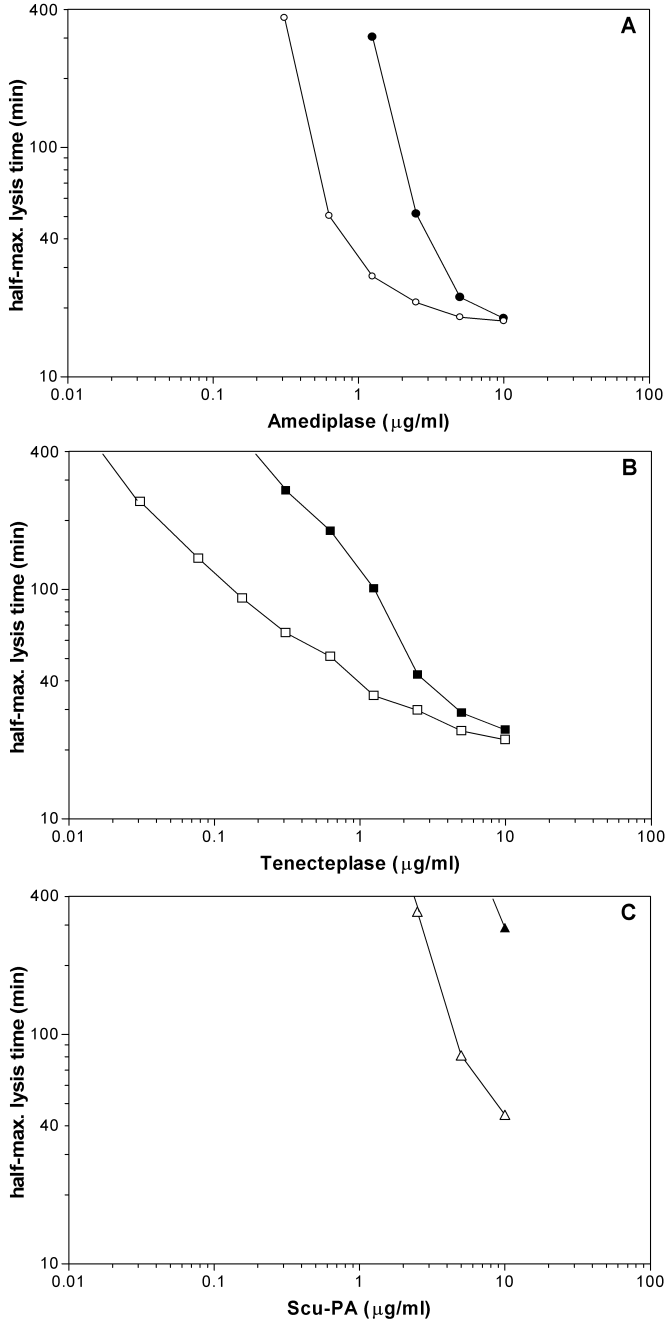

Figure 3. Half-maximal lysis times obtained for increasing concentrations of Amediplase (A), Tenecteplase (B) and scu-PA (C) with stirring, using an external plasma clot lysis model sensitive to TAFI. (mean, n = 6) Half-maximal lysis times were determined from the clot lysis profiles with (*open symbols*) or without (*closed symbols*) a specific TAFIa inhibitor (PCI) and plotted against the PA concentration.

In the presence of PCI (Fig.4A), Amediplase was more effective than Tenecteplase at PA concentrations between 1-10 μg/ml (1.25 μg/ml, p = n.s.; 2.5 μg/ml, p = 0.009; 5 μg/ml, p = 0.009 and 10 μg/ml, p = 0.04) while, at lower concentrations, Tenecteplase was more effective than Amediplase. This corresponded well to the results in the first model that was insensitive to TAFI (Fig.2). A similar external plasma clot lysis model sensitive to TAFI was used to confirm the results obtained in the presence of PCI. Plasma clots were prepared without addition of FITC-labelled fibrinogen and external lysis was performed with 5 μg/ml of each

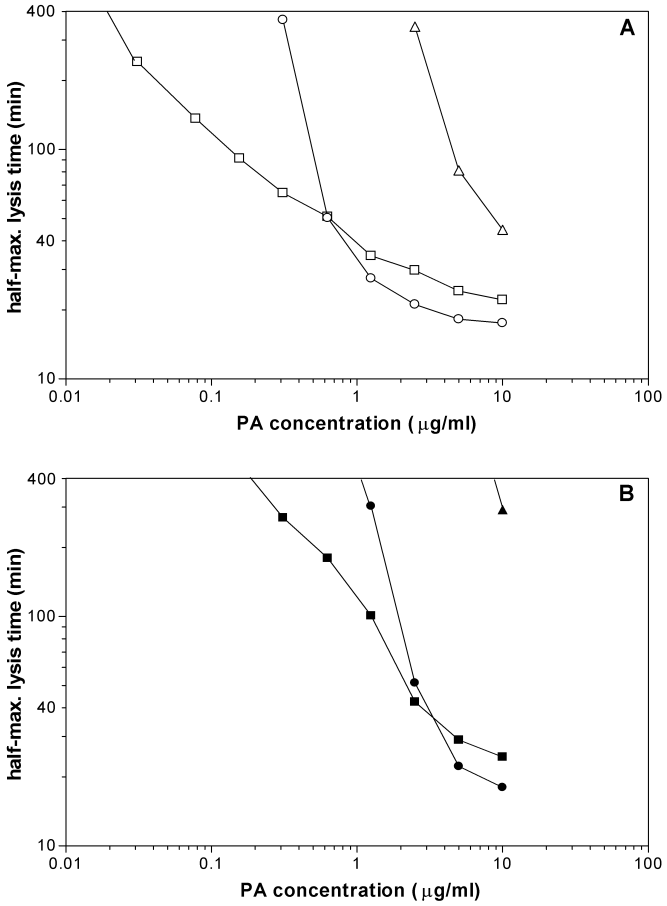

Figure 4. Comparison of the half-maximal lysis times obtained for Tenecteplase (squares), Amediplase (circles) and scu-PA (triangles) in the absence (A) or presence (B) of PCI with stirring, using an external plasma clot lysis model sensitive to TAFI. Differences between half-maximal lysis times of Tenecteplase and Amediplase were tested with an unpaired t-test. *p* values <0.05 were considered statistically significant. **A**, 1.25 μg/ml, *p* = n.s.; 2.5 μg/ml, *p* = 0.009; 5 μg/ml, *p* = 0.009 and 10 μg/ml, *p* = 0.04. **B**, 5 μg/ml, *p* = 0.05 and 10 μg/ml, *p* = n.s..

PA, as earlier described. After 30 min plasma clot lysis was stopped and the fibrin content in the remaining plasma clot determined. The plasma clots subjected to external lysis mediated by Tenecteplase contained 51.6 ± 3.1 % (mean ± SD, n = 4) of the initial fibrin content while the plasma clots treated with Amediplase contained 13.6 ± 7.3 % of the initial fibrin content (p = 0.008), corroborating the differences observed in the model containing FITC-labelled fibrinogen.

In the absence of PCI (Fig.4B) Amediplase still appeared to be more effective than Tenecteplase at 5 and 10 μg/ml but this did not reach statistical significance (5 μg/ml, p = 0.05 and 10 μg/ml, p = n.s.). This confirmed that Amediplase was slightly more sensitive to TAFIa than Tenecteplase. scu-PA was less effective than Amediplase or Tenecteplase.

Discussion

In this study, we compared the lytic activity of Tenecteplase, Amediplase and scu-PA in external plasma clot lysis models taking into account factors that may influence clot dissolution such as TAFIa activity, thrombin generation in the exterior plasma, and stirring conditions.

In the first model, we showed that Amediplase was more active than Tenecteplase at therapeutic concentrations. For lower concentrations, Amediplase exhibited a lower lytic activity than Tenecteplase (Fig.2). The activity of scu-PA was clearly lower than that of both Tenecteplase and Amediplase. The addition of hirudin to the exterior plasma did not lead to differences in the lysis rates, which probably reflects negligibly low thrombin generation in citrated plasma without recalcification.

Thereafter, we investigated the inhibitory effect of TAFIa on these three PAs using an external plasma clot lysis model modified for this purpose. In this model, plasma clots were immersed in recalcified plasma to allow the activation of TAFI. This exterior plasma was defibrinated by treatment with batroxobin, a snake-derived enzyme, which leads to fibrin formation but not to TAFI activation (20,22). Thrombomodulin was added to the defibrinated plasma and experiments were performed at room temperature instead of 37°C to enhance the activation of TAFI and the stability of TAFIa, respectively.

The addition of a specific TAFIa inhibitor increased the percentage of lysis under all conditions corroborating an inhibitory effect of TAFIa on all PAs tested, both with (Fig.3) and without stirring. Previously, we compared the inhibitory effect of TAFIa on seven different PAs using an internal lysis model, in which the PA is incorporated into the plasma clot (18). TAFIa was able to inhibit internal lysis mediated by all PAs tested and a dependence on the type and concentration of the PA used was observed. The inhibitory effect of TAFIa strongly decreased at high PA concentrations. Recently, Cruden et al (23) also showed that tPA-mediated fibrinolysis was accelerated by the inhibition of TAFIa when tPA concentrations supplemented to blood corresponded to plasma concentrations in the range of 0.1-0.2 µg/ml but not when higher tPA concentrations (1-5 µg/ml) were used. A concentration-dependence was also observed for the effect of TAFIa in the external lysis model, described here. The inhibitory effect of TAFIa was high at low PA concentration and decreased with increasing PA concentrations (Fig.3). In the therapeutic range (5-10 µg/ml), the TAFIa effect was negligible for

both Amediplase and Tenecteplase. This agreed with previous findings for Alteplase using in vitro external lysis (24) and in vivo thrombolysis (25) models. Using model thrombi, Mutch et al. (26) observed a significant effect of TAFIa in external lysis mediated by two chain u-PA, scu-PA and tPA when the model thrombi were surrounded by plasma (30%). Interestingly, the inhibitory effect of TAFIa became less obvious by increasing the plasminogen concentration in the surrounding plasma. All findings concur with the fact that the loss of the TAFIa effect coincides with plasminogen or PA concentrations at which a high plasmin concentration is achieved. It is thus possible that these high plasmin concentrations inhibit TAFI and TAFIa by proteolytic cleavage (27). In addition, the loss of fibrin specificity at therapeutic concentrations makes plasminogen activation less dependent on the availability of C-terminal lysines on fibrin, as suggested by Colucci et al. (24). Mutch et al. did not observe a decreased TAFIa effect at high PA concentrations (26). Their highest PA concentration was, however, 1 µg/ml, i.e. 10-fold lower than the highest PA concentration in our study.

In addition to Tenecteplase and Amediplase, scu-PA represents a potentially new thrombolytic agent. In the PROACT trial, the safety and recanalisation efficacy of recombinant pro-urokinase (rpro-UK), Prolyse, in acute ischaemic stroke was investigated (28). In the models used here, scu-PA was less effective than the other two PAs and a TAFIa effect was seen even at 10 µg/ml scu-PA. When comparing the results for scu-PA in the first external lysis model with those obtained in the second external lysis model in the presence of PCI, we observed a further decrease in lytic activity in the second model. It is likely that the presence of thrombomodulin in the second external lysis model increases the direct inactivation of scu-PA by thrombin (10,11).

We conclude that Amediplase was slightly more active than either Tenecteplase or scu-PA at therapeutic concentrations under all conditions (with/without hirudin, with/without TAFIa activity and with/without stirring) using two different external clot lysis models. In a phase II clinical trial, over 75% of the patients with acute myocardial infarction treated with Tenecteplase achieved coronary artery patency (TIMI 3 flow grade) at 90 min after administration, corresponding to an average plasma concentration of 3.6 µg/ml during this 90 min interval (29,30). In acute myocardial infarction patients, administration of Amediplase achieved a similar TIMI 3 flow grade at 60 min (3K2 trial) (31). For Amediplase higher weight-adjusted doses were used in comparison to Tenecteplase and the average

plasma concentration during treatment was 7.9 µg/ml (Menarini Ricerche, *unpublished*). Our results (Fig.2 and 4) show that this is the concentration range where Amediplase was slightly more active than Tenecteplase resulting in a lower half-maximal lysis time.

Finally, the role of TAFI during optimal and successful thrombolytic therapy is probably low. Nevertheless, depending on the thrombus composition and architecture, haemodynamic conditions and distinct physiological interactions of plasminogen and PAs in the vascular environment, it is conceivable that the local PA concentration does not reach optimal levels creating conditions, in which TAFI might affect the lysis rates during thrombolytic therapy.

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CHAPTER 8

TAFI and pancreatic carboxypeptidase B (CPB) modulate *in vitro* capillary tube formation by human microvascular endothelial cells.

(submitted for publication)

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Summary

Besides having a key role in fibrinolysis, the plasminogen system has also been implicated in cell migration and angiogenesis. A common mechanism is the binding of plasminogen to carboxy-terminal lysine residues in partially degraded fibrin or on cellular surfaces. Here we examined the involvement of TAFI, a plasma procarboxypeptidase B, and pancreatic CPB in capillary tube formation using an *in vitro* system, which was largely plasminogen-dependent. Human microvascular endothelial cells (hMVECs) were seeded on a 3D plasma clot matrix and subsequently stimulated with bFGF/TNF-α. Tube formation was analyzed and fibrin degradation products (FbDP) were determined in the medium. Supplementation of the matrix with additional TAFI or with CPB produced a reduction in tube formation. Pretreatment of hMVECs with CPB before seeding resulted in a similar effect. FbDP-levels indicated a concomitant reduction in matrix proteolysis. The addition of a TAFIa inhibitor increased tube formation and FbDP release into the medium. In a wound assay, CPB impaired the migration of hMVECs in a dose-dependent manner. Overall, these results demonstrate that TAFI and CPB in these systems modulate the plasminogen system both in the matrix and on the cell surface, thus leading to the inhibition of endothelial cell movement and tube formation.

Introduction

Activated thrombin activatable fibrinolysis inhibitor (TAFIa) is a basic carboxypeptidase that inhibits fibrinolysis by preventing the positive feedback in plasmin generation. The proenzyme TAFI, is synthesized in the liver and present in plasma and can be activated by trypsin, plasmin and thrombin [1,2] by a single cleavage at Arg-92. The efficiency of TAFI activation by thrombin is increased by the endothelial cell receptor thrombomodulin [3]. In addition, the activation of TAFI by plasmin is stimulated by glycosaminoglycans [4], which are synthesized by endothelial cells. Activated TAFI (TAFIa), also described as plasma carboxypeptidase B, carboxypeptidase U and carboxypeptidase R [1,5-7], is intrinsically unstable [8] with a half-life of about 8 min at 37°C [9]. TAFIa cleaves carboxy-terminal basic residues from proteins with a preference for carboxy-terminal arginine residues over carboxy-terminal lysine residues [5]. Pancreatic carboxypeptidase B (CPB), a digestive basic carboxypeptidase displays high homology with TAFI [10] but in contrast to TAFIa, CPB is a stable

protease.

The conversion of plasminogen into active plasmin is initiated either by the tissue-type plasminogen activator (tPA) or the urokinase-type plasminogen activator (uPA). tPA is mainly involved in the dissolution of fibrin in the circulation and uPA in the induction of pericellular proteolysis [11]. The interaction between plasminogen and fibrin is dependent on lysine binding sites on plasminogen. Plasmin is able to generate new carboxy-terminal lysine and arginine residues in fibrin enhancing its own binding as well as the binding of plasminogen [12]. This enhanced binding results in an increased catalytic efficiency of plasmin formation [13,14] and can be blocked by TAFIa [15]. TAFIa was shown to inhibit tPA and uPA *in vitro* plasma clot lysis [16,17]. In addition, *in vivo* inhibition of TAFIa by a specific inhibitor, i.e. potato carboxypeptidase inhibitor (PCI), was shown to enhance tPA-induced thrombolysis [18-22].

Besides the key role that these carboxy-terminal lysine residues play in fibrinolysis they have also been implicated in cell migration, wound healing and angiogenesis where they function as binding sites for plasminogen [23]. Lysine analogs, such as epsilon-amino-caproic acid (ε-ACA) and tranexamic acid (Cyclokapron) efficiently prevent plasmin formation [24-26] and inhibit tumor cell metastasis and primary tumor growth [27,28]. Moreover, binding of plasminogen to the cell surface can be abrogated by treatment with pancreatic CPB as well as with TAFIa [29].

It is therefore possible that TAFI functions as a broad modulator of the plasminogen system in its various functions. Although an $TAFI^{-/-}$ mice did not present an overt phenotype [30], recently Swaisgood et al. [31] demonstrated an *in vivo* role for TAFI as a modulator of the plasminogen system during fibrinolysis and during cell migration. Furthermore, te Velde et al. [32] showed that TAFI-deficient mice present an impaired healing of cutaneous wounds and of colonic anastomoses. However, little is known about the possible effects of TAFI on endothelial cell migration and neovascularization.

Here, we investigated the participation of TAFI in the formation of capillary-like tubular structures *in vitro* using a model for tube formation that relies mainly on the plasminogen/uPA system [33,34]. The addition of antibodies against uPA, aprotinin or uPA receptor (uPAR) completely inhibited bFGF/TNF-α stimulated tube formation, while the addition of anti-tPA antibody or of a general MMP-inhibitor resulted only in a moderate inhibition. Frequently,

neovascularization occurs in adults under conditions, in which a fibrinous exudate is formed and this can facilitate the angiogenesis process [35]. In this study, we sought to mimic the formation of capillary-like structures *in vitro* using a three-dimensional plasma clot matrix covered by human microvascular endothelial cells (hMVECs).

To our knowledge, this is the first time that TAFI has been demonstrated to have an effect on *in vitro* capillary-like tube formation. Moreover, this effect could not be solely ascribed to the cleavage of carboxy-terminal lysine residues from partially degraded fibrin, which composes the known substrate for TAFIa and rather points to the existence of additional physiological substrates. We propose that TAFI might be involved in neovascularisation processes during thrombus resolution, wound healing and atherosclerosis [31,32,36,37].

Materials and Methods

Materials

Cell culture reagents were purchased as previously described [34]. Human serum (HS) was obtained from a local bloodbank and was prepared from freshly obtained blood from 10-20 healthy donors, pooled and stored at 4°C. Newborn calf serum (NBCS) was obtained from Life Technologies (Grand Island, NY, USA). NBCS and HS were heat-inactivated before use. Basic fibroblast growth factor (bFGF) was purchased from Prepro Tech EC (London, UK) and human recombinant tumor necrosis factor-α (TNF-α) was a gift from Dr. J. Travernier (Biogent, Gent, Belgium) and contained 2.45 x 10^7 U/mg protein and less than 40 ng lipopolysaccharide per mg protein. Aprotinin was purchased from Pentapharm Ltd. (Basel, Switzerland). Heparin and bovine thrombin were obtained from Leo Pharmaceutics Products (Weesp, the Netherlands) and potato carboxypeptidase inhibitor (PCI) was acquired from Calbiochem (La Jolla, CA, USA). Pancreatic carboxypeptidase B was purchased from Sigma-Aldrich (St. Louis, MO, USA) and TAFI was isolated as described previously [38]. Citrated platelet-poor plasma from ten healthy volunteers was obtained from the blood bank, pooled and stored at -80°C. TAFI-depleted plasma was prepared using an anti-TAFI IgG sepharose column essentially as previously described [39]. The monoclonal u-PA receptor (uPAR)-blocking antibody H-2 was a kind gift from Dr. U. Weidle (Boehringer Mannheim, Penzberg, Germany) [40]. Rabbit polyclonal

anti-u-PA antibodies were prepared in our laboratory [41]. Horseradish peroxidase (HRP) conjugates of sheep anti-human TAFI IgG and rat anti-mouse IgG were from Affinity Biologicals (Hamilton, Ontario, Canada) and DAKO (Glostrup, Denmark), respectively. The CD31 murine IgG (clone 1A10) was from Monosan (Sanbio, Uden, The Netherlands).

Cell culture

Human foreskin microvascular endothelial cells (hMVECs) were isolated, cultured and characterized as previously described [42,43].

Preparation of the three-dimensional (3D) plasma clot matrix

Plasma clot matrices were prepared by the addition of 1 U/mL thrombin to citrated platelet-poor plasma. Immediately afterwards, 300 µL aliquots of this mixture were added to the wells of a 48-well plate. The plasma clot matrices were left at room temperature for at least 30 minutes and then equilibrated at 37ºC, under humidified 5% CO_2/ 95% air atmosphere with serum-containing culture medium (Medium 199 - M199, supplemented with 10% (v/v) HS, 10% (v/v) NBCS, 100 IU/ml penicillin and 0.1 µg/ml streptomycin). During the next 24 hours, the matrices were thoroughly washed with serum-containing culture medium (3 to 4 times).

Pre-treatment of hMVECs with CPB

The hMVECs were detached by treatment with trypsin/EDTA and suspended in serum-containing culture medium. After washing, the hMVECs were incubated with 50 units/ml of pancreatic CPB in serum-containing culture medium at 4°C. After 30 min the CPB-containing medium was removed and the hMVECs were washed by centrifugation. These pre-treated hMVECs were resuspended in serum-containing culture medium and seeded at a split ratio of 2:1 on top of the plasma matrices. Non-treated cells were submitted to the same procedure with serum-containing medium, in the absence of CPB.

In vitro tube formation assay

The formation of capillary-like tubes was evaluated essentially as previously described in fibrin matrices [33]. Confluent hMVECs were seeded at a split ratio of 2:1 on top of the plasma matrices and cultured growth factor free for 24 hours in serum-containing culture medium. Then, the cells were stimulated with stimulation medium composed of serum-containing culture

medium supplemented with bFGF (10 ng/ml) and TNF-α (10 ng/ml). The reagents to be tested were either added to plasma before preparing the matrix or added to the stimulation medium. Every second day the stimulation medium was collected and renewed. After seven days the formation of tubular structures was analyzed by phase-contrast microscopy. Quantification of the length of the structures formed was performed essentially as previously described [33], by measuring in six randomly chosen microscopic fields (7.3 mm^2/field) using a Nikon FXA microscope equiped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software.

Wound assay [44]

Confluent hMVECs were seeded on fibronectin-coated dishes of a 48-wells plate in serum-containing culture medium with 150 µg/ml crude endothelial cell growth factor (ECGF) and 5 U/ml heparin. Subsequently, hMVECs were growth factor deprived for 24 hours. A scratch was applied to the confluent monolayer and the cells were washed for three times with M199 to remove the detached cells. Immediately, the stimulation medium (serum-containing culture medium with 10 ng/ml bFGF and 10 ng/ml TNF-α) supplemented with the compounds of interest was added to the wells and the cells were incubated at 37ºC, under humidified 5% CO_2/ 95% air atmosphere. During the following 24h, photographs of the wound were taken every 2h and wound analyses were performed by calculating the diameter of the wound for each time point (4 measurements/well) for duplicate wells.

Proliferation assay

hMVECs were seeded 1:8 on a fibronectin-coated 24-well plate in serum-containing culture medium (0.5 ml/well). After 24 hours, the cells were stimulated with bFGF (10 ng/ml) alone or in combination with CPB (1, 10 or 100 U/ml) and incubated at 37°C under humidified 5% CO_2/ 95% air atmosphere for 96 hours. After the incubation period, the cells were washed for three times with M199 with 100 IU/ml penicillin and 0.1 mg/ml streptomycin and fixed for 10 minutes with 2% glutaraldehyde. The cell membrane was rendered permeable by fixation with 70% ethanol for 10 minutes. The cells were stained with crystal-violet for 2h, washed for four times with MilliQ and dried overnight at room temperature. Cell counting was performed by the computer program Optimas. The experiment was performed in duplicate and the data was based on six countings per well.

Fibrin degradation products (FbDP) EIA and uPA ELISA

The FbDP EIA was performed essentially as previously described [45]. Calibration was performed using whole-blood clot lysate [46] and pooled normal plasma was used as control. FbDP levels were calculated from duplicate measurements.

uPA antigen was measured essentially as previously described [33].

Immunohistochemistry

Tissue sections (5 µm) of human atherosclerotic plaques with an incorporated mural thrombus were dewaxed by immersion in xylene and rehydrated in decreasing concentrations of ethanol. For HPS staining, the sections were counterstained with Mayer's hematoxylin, phloxin and saffron. After dehydration in a reversed ethanol-xylene series, the sections were prepared for microscopy. Inhibition of endogenous peroxidase was accomplished by immersion in 1% hydrogen peroxidase in absolute methanol for 20 min. The sections were washed in deionized water and equilibrated in phosphate-buffered saline (PBS, pH 7.4). For antigen retrieval, sections were incubated in 0.1 M sodium-citrate in a microwave at 700 Watt until boiling point was reached, followed by a period of 10 min at 180 Watt. Subsequently, all the sections were blocked by 5% BSA in PBS to prevent nonspecific binding. Different antibodies were applied to the sections overnight at 4ºC. After washes with PBS, the sections were exposed to the second antibody, the biotinylated horse anti-mouse IgG diluted in 1% BSA/PBS in a concentration of 1:400, for 1 hour at room temperature. Then after further washes with PBS, the sections were incubated for 30 min at room temperature with the HRP Avidin Biotinylated Complex (ABComplex). The signal was amplified by biotinylated tyramides as described [47] for 10 min at room temperature, followed again by the HRP ABComplex for 30 min at room temperature. The sections were washed with PBS and stained with Novared for a period of 5 to 10 min. Finally, the sections were washed in aquadest, counterstained with hematoxylin, washed in running water and dehydrated in a reversed ethanol-xylene series and prepared for microscopy.

Statistical analysis

Tube formation results were expressed as the mean percentage ± SEM of the results obtained in the bFGF/TNF-α condition. For statistical analysis, we used one-way ANOVA followed by the Dunnett's test as post-test. The Dunnett's test is a modified t-test that takes into account multiple comparisons with a control condition. Statistical significance was accepted at $P<0.05$.

Results

Involvement of TAFI in tube formation in a 3D plasma clot matrix

To study the involvement of TAFI during tube formation, we used a 3D *in vitro* model where a plasma clot is used to mimic the provisional wound matrix. hMVECs were seeded on top of the plasma clot matrix and stimulated with bFGF and TNF-α to form capillary-like structures (Fig.1A). Figure 1B demonstrates that addition of the TAFIa-specific inhibitor PCI stimulated the formation of tubular structures by 57% ($p<0.01$) while increasing TAFI concentration (by 50 nM) in the plasma clot matrix inhibited tube formation (42%, $p<0.05$). In agreement with previous findings, the plasma inhibitor aprotinin caused a strong inhibition of tube formation (80%, $p<0.01$) confirming the dependency of the model on the activity of plasmin. Similarly, inhibition of uPA by anti-uPA and of uPAR by anti-uPAR (MoAb H2) also reduced tube formation corroborating the involvement of cell-bound u-PA on tube formation (both over 80%, $p<0.01$) (*not shown*). Pancreatic CPB was incorporated into the plasma clot matrix to compare the effect of this active and stable basic carboxypeptidase with that of TAFI. Addition of CPB (1-10 units/ml) to the matrix induced a significant and dose-dependent inhibition of tube formation, which was significant at 5 and 10 units/ml CPB (both $p<0.01$; Fig.1C).

The inhibitory effect of TAFI during tube formation might emerge from its known downregulation of fibrin matrix degradation or from an effect on hMVECs themselves. To further explore this, the hMVECs were pre-treated with CPB. Subsequently, the hMVECs were washed and seeded on top of the plasma clot matrix. After 24 h they were stimulated with bFGF/TNF-α alone (CPB_{+}) or combined with the addition of CPB to the medium (25 units/ml, CPB_{++}). This pre-treatment (CPB_{+}) resulted in an inhibition of tube formation (58%, $p<0.01$) pointing to a direct effect on the hMVECs (Fig.1C). Furthermore, when CPB was also added to the stimulation medium (CPB_{++}) a supplementary inhibition in tube formation was observed (73%, $p<0.01$). It is interesting to notice that either supplementation of the matrix with 10 units/ml CPB or treatment of hMVECs with CPB in combination with CPB in the medium (CPB_{++}) resulted in an inhibition of tube formation comparable to the addition of aprotinin. This suggests that the observed effects reflected complete interference with the uPA/plasmin system.

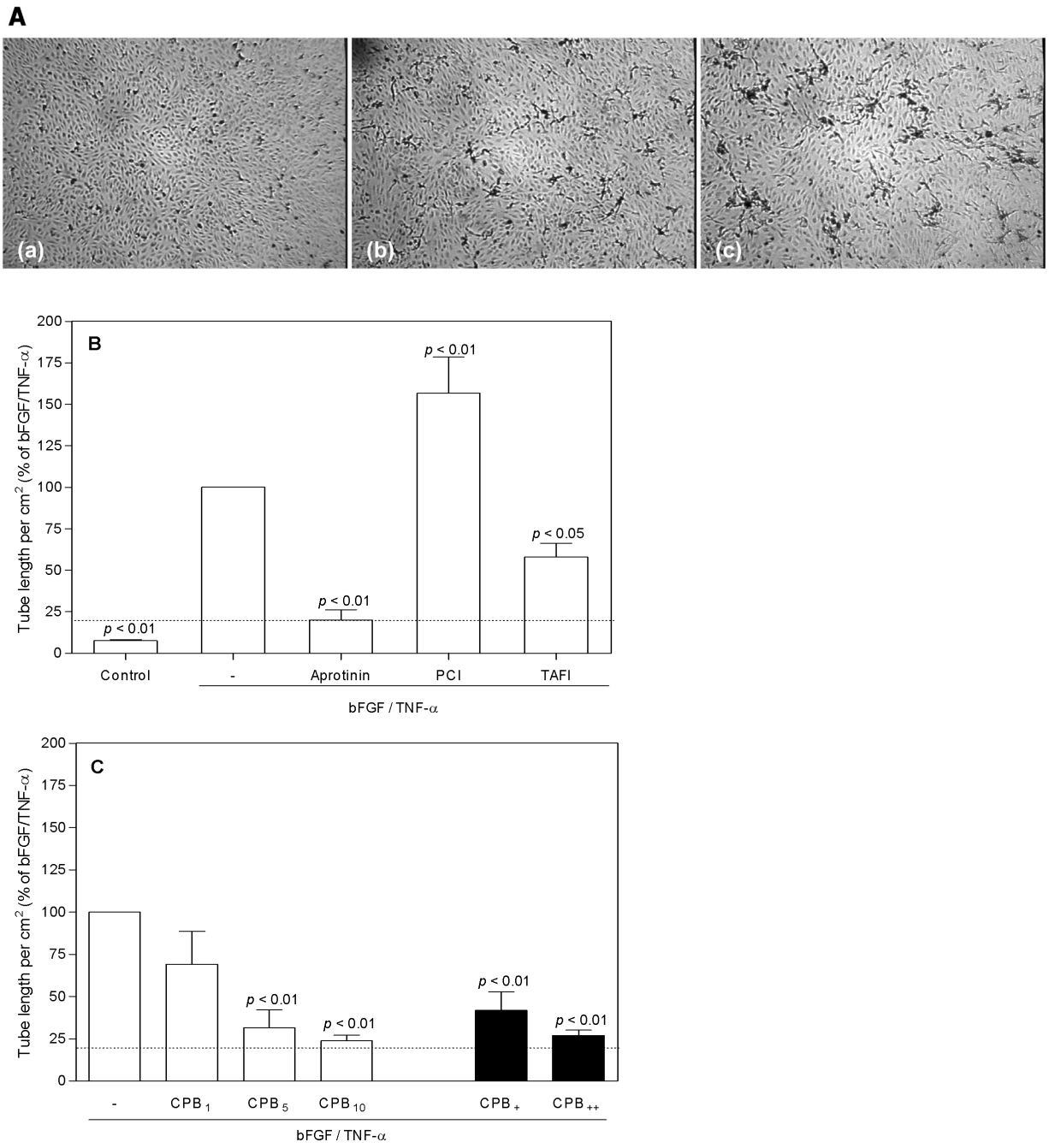

Figure 1. Formation of capillary-like structures in a 3D plasma clot matrix. hMVECs were cultured on top of a 3D plasma clot matrix in serum-containing culture medium. (**A**) Phase-contrast photomicrographs of tube formation with stimulation with bFGF (10 ng/ml) and TNF-α (10 ng/ml) were made at **(a)** day 1, **(b)** day 3 and **(c)** day 6 (original magnification 20x). (**B**) hMVECs were cultured with and without stimulation with bFGF and TNF-α. Additionally, purified TAFI (50 nM) or potato carboxypeptidase inhibitor (PCI, 30 µg/ml) was added to the plasma clot matrix. Aprotinin (200 KIU/ml), a plasmin inhibitor, was added to the stimulation medium. (**C**) Pancreatic carboxypeptidase B (CPB) was either incorporated into the plasma clot matrix (CPB 1, 5, 10 units/ml) or hMVECs were pre-treated with CPB (CPB_+) or pre-treated with CPB and 25 units/ml CPB added to the medium (CPB_{++}). After 6 days of culture the tube length per cm^2 was expressed as % of the bFGF/TNF-α control as described. The data represent mean percentage ± SEM of 3 independent experiments performed in duplicate wells. The dotted line indicates the extent of tube formation that can be inhibited by a plasmin inhibitor, aprotinin.

Fibrin degradation products (FbDPs) accumulation during tube formation

The amount of FbDPs accumulated in the conditioned medium markedly increased during tube formation, as evaluated during two 48h periods after initial stimulation by bFGF/TNF-α (Fig.2A). This accumulation was largely inhibited by aprotinin (81%, $p<0.01$). Addition of PCI enhanced FbDP accumulation in the medium (87%, $p<0.01$) whereas addition of TAFI (Fig.2A) inhibited the release of FbDPs compared to bFGF/TNF-α stimulation only (57%, $p<0.01$). Moreover, when the matrix was supplemented with CPB (Fig.2B), FbDP levels decreased (71% CPB_1, 86% CPB_5 and 89% CPB_{10}, all $p<0.01$) pointing to a downregulation of fibrinolysis. Pre-treatment of hMVECs with pancreatic CPB also inhibited FbDPs release (87% CPB_+ and 93% CPB_{++}, both $p<0.01$).

uPA accumulation during tube formation

The accumulation of uPA in the stimulation medium during tube formation was significantly decreased when hMVECs were pre-treated with CPB (46%

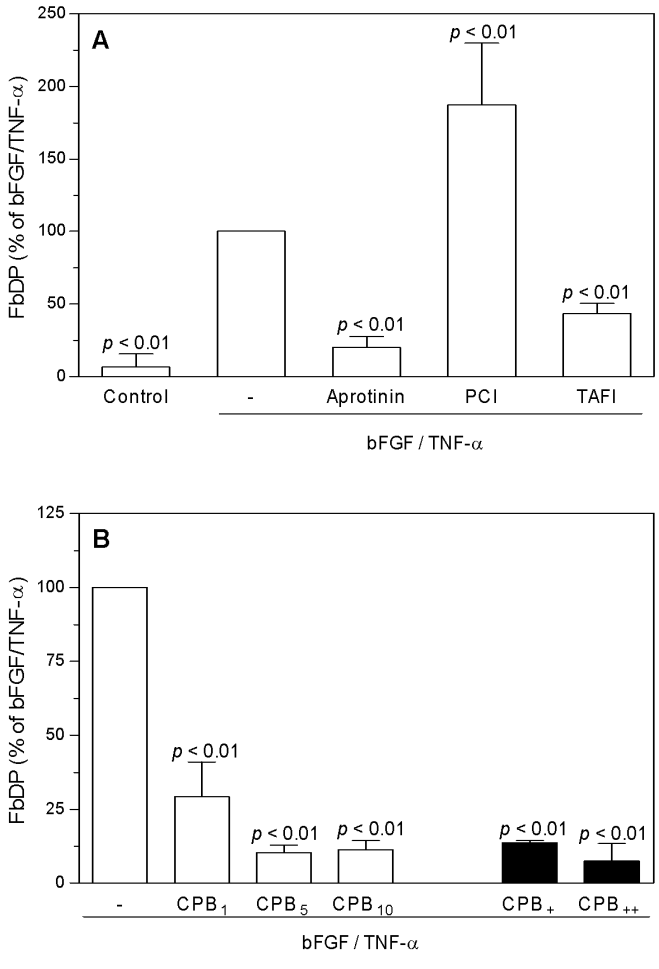

Figure 2. Release of fibrin degradation products (FbDP) during the formation of capillary-like structures in a 3D plasma clot matrix. The stimulation medium was renewed at 48 h intervals and the conditioned media of the first two stimulation periods were collected for assay of FbDPs. The two values were added to obtain the FbDP accumulatioon over the 96 h period. (**A**) hMECs were cultured with and without stimulation with bFGF/TNF-α. In addition, the matrix was supplemented with PCI (30 µg/ml), aprotinin (200 KIU/ml) or additional purified TAFI (50 nM) where stated. (**B**) CPB was either incorporated into the plasma clot matrix (CPB 1, 5, 10 units/ml) or hMVECs were pre-treated with CPB (CPB_+) or pre-treated with CPB and 25 units/ml CPB added to the medium (CPB_{++}). The data represent mean ± SEM of 3 independent experiments performed in duplicate wells. The FbDP level for the bFGF/TNF-α condition corresponds to 30 µg/ml).

CPB_+ and 48% CPB_{++} compared to bFGF/TNF-α, both $p<0.01$) (*not shown*). The amount of uPA found in the medium under bFGF/TNF-α stimulation corresponded to about 16 ng/ml. The addition of CPB, PCI or TAFI to the matrix, did not alter the uPA accumulation compared to bFGF/TNF-α.

TAFI concentration in the plasma clot matrix and consequences for tube formation

We examined the effect of reducing TAFI concentration in the plasma clot matrix by testing serial dilutions of normal pooled plasma in TAFI-depleted plasma. Reduction of the TAFI content of the plasma clot matrix caused an acceleration of the tube formation (Fig.3A) and an increase in FbDP accumulation (Fig.3B). Depletion of TAFI in the plasma clot matrix or addition of PCI to normal plasma clot matrix had similar results (Fig.3A,B).

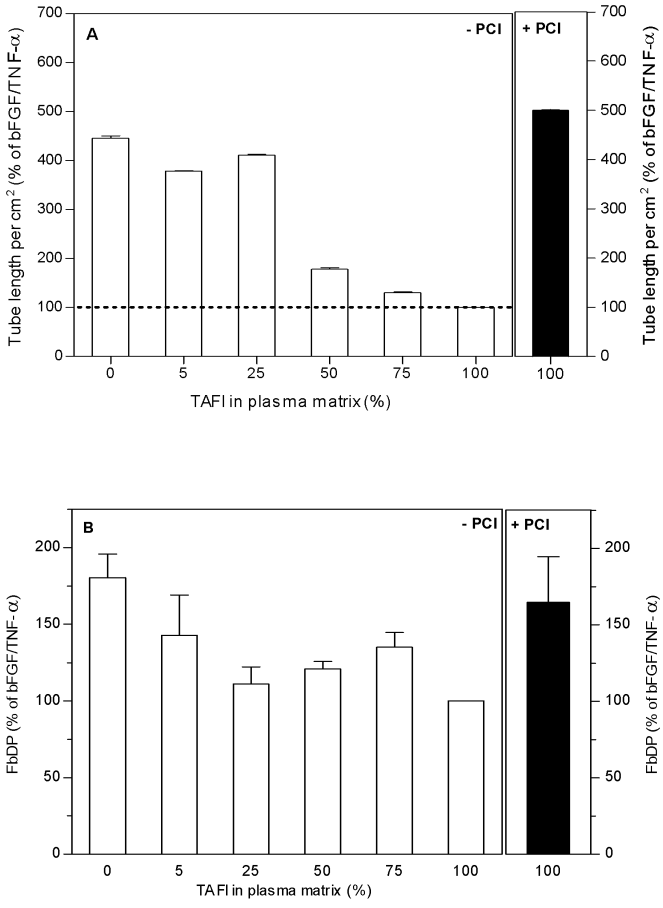

Figure 3. Effect of decreasing TAFI concentrations in the plasma clot matrix on the tube formation. Serial dilutions of normal plasma in TAFI-depleted plasma were performed and these mixtures were used to prepare the 3D plasma clot matrix (*open bars*). hMVECs were cultured on top of this matrix and stimulated with bFGF (10 ng/ml) and TNF-α (10 ng/ml). Additionally, potato carboxypeptidase inhibitor (PCI - 30 µg/ml) was added to the plasma clot matrix where stated (*black bar*). (**A**) After 6 days of culture the tube length per cm^2 was expressed as mean percentage of the bFGF/TNF-α control ± SEM of a representative experiment. (**B**) Release of FbDP during the formation of capillary-like structures in a 3D plasma clot matrix. The stimulation medium was renewed at 48 h intervals and the conditioned media of the first two stimulation periods were collected for assay of FbDPs. The two values were added to obtain the FbDP accumulatioon over the 96 h period. The data represent the mean percentage of the bFGF/TNF-α control ± SEM of 3 independent experiments (FbDP level for the bFGF/TNF-α condition corresponds to 5 µg/ml). The dotted line indicates the amount of tube formation under normal conditions, i.e. plasma containing the normal amount of TAFI.

The reduction of TAFI content not only caused an increase in capillary tubes but also modified their structure. The tubes formed in matrices containing less TAFI displayed a more extensive network and were accompanied by wider tubes, often in the upper area of the plasma clot matrix, suggesting increased fibrin degradation (Fig.4A-C) and also possibly an altered migration.

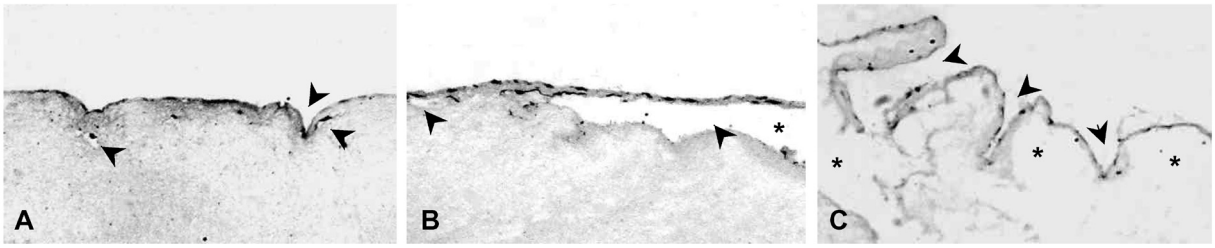

Figure 4. Formation of capillary-like tubular structures by hMVECs at decreasing TAFI concentrations. hMVECs were cultured on top of a 3D plasma clot matrix and stimulated with bFGF (10 ng/ml) and TNF-α (10 ng/ml). The plasma clot matrices were prepared by serial dilutions of normal plasma in TAFI-depleted plasma. Histological cross-sections of the plasma clot matrices containing 75% (**A**), 5% (**B**) and 2.5% (**C**) of TAFI. Tubular structures are indicated by arrowheads and the asterisks indicate areas where extensive lysis of the plasma clot matrix has occurred, original magnification 40x.

CPB affects the migration of hMVECs in a concentration dependent way

Pre-treatment of hMVECs with CPB resulted in the inhibition of tube formation (Fig.1). Moreover, decreasing TAFI concentrations in plasma resulted not only in an increase in fibrinolysis (Fig.3B) but also in an increased hMVECs infiltration (Fig.3A; Fig.4). These results suggest that these basic carboxypeptdases are able to modulate hMVECs functions, probably via the regulation of the uPA/plasmin system. We therefore used CPB to study the effect of basic carboxypeptidase activity on the proliferation and migration of hMVECs. The proliferation of hMVECs was not affected in the presence of CPB (1, 10 and 100 U/ml), as estimated by cell counting (*not shown*). A wound assay was used to investigate the effect of carboxypeptidase B activity on hMVECs migration under similar stimulation conditions as used for the tube formation assay, namely stimulation by bFGF/ TNF-α. In this assay the migration of hMVECs was impaired in a dose-response manner by increasing CPB concentrations in a 24-hour period (Fig.5). The uPA/ plasminogen system was also involved during the migration of hMVECs, under bFGF/TNF-α stimulation, as the addition of anti-uPAR, anti-uPA or aprotinin delayed the migration of hMVECs.

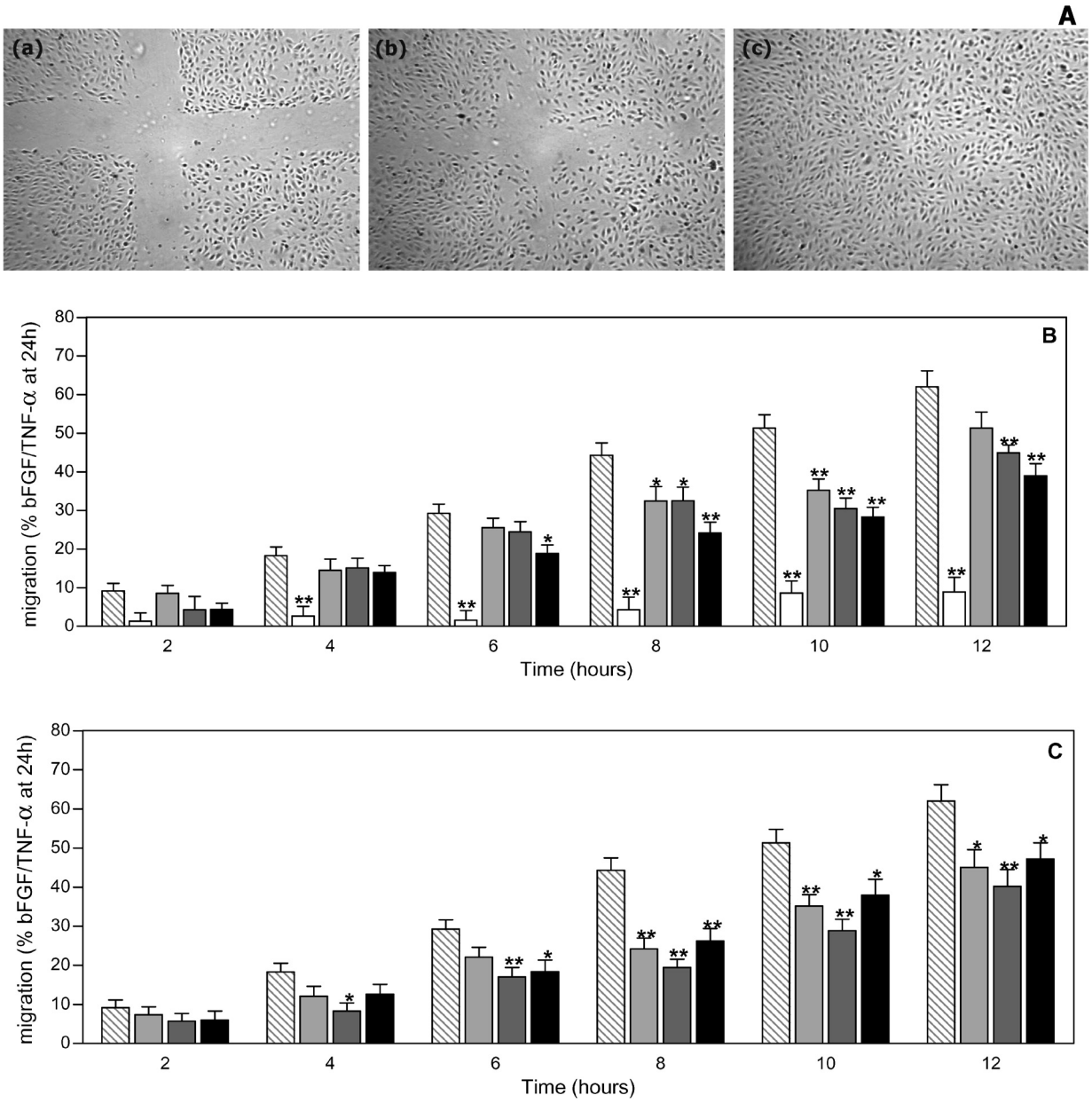

Figure 5. Pancreatic carboxypeptidase B inhibits migration of hMVECs in a concentration-dependent way. Confluent hMVECs were seeded on a fibronectin coated well to obtain a cobblestone monolayer. (**A**) Scratches were made through the monolayer and photomicrographs of the migrating hMVECs were taken at regular intervals. **(a)**, 0 hours; **(b)**, 12 hours and **(c)**, 24 hours (original magnification 20x). (**B,C**) Immediately after wounding, the cells were stimulated with bFGF (10 ng/ml) and TNF-α (10 ng/ml) (*striped bars*). (**B**) A negative control without stimulation was included (*open bars*). Some wells were exposed to combined stimulation with bFGF/TNF-α and increasing concentrations of CPB (**B**) 1 unit/nl (light grey bar), 10 units/ml (*dark grey bar*) and 100 units/ml (*black bar*) or (**C**) anti-uPA antibody (30 µg/ml) (*black bar*), anti-uPAR antibody (25 µg/ml) (*dark grey bar*) and aprotinin (200 U/ml) (*light grey bar*). Data represent mean ± SEM of 3 experiments performed in duplicate wells. P-values for comparisons between the different compounds tested and the bFGF/TNF-α condition were calculated by ANOVA with the Dunnett's test as post-test; * $P<0.05$ and ** $P<0.01$.

Localization of TAFI in an atherosclerotic plaque

To investigate the presence and localization of TAFI in new vascular structures formed in a fibrinous environment, immunohistochemical analysis of TAFI was performed in tissue sections of atherosclerotic plaques with organized thrombi. The neointima with incorporated thrombus contained new capillaries. The endothelial cells of the newly formed microvessels were visible after staining for CD31 (PECAM-1) (Fig.6A). Staining with an antibody against TAFI (Fig.6B,C) revealed that TAFI was, as expected, present in the fibrinous exudate, and accumulated in many of the vascular structures in this thrombus. This occurred possibly by the colocalization of TAFI with the endothelial cells lining the vessels.

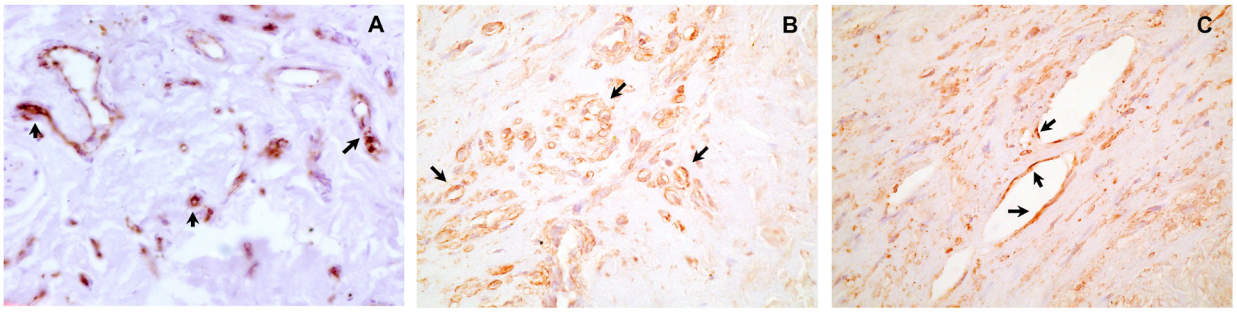

Figure 6. Localization of TAFI and endothelial cell marker CD31 (PECAM-1) in neovessels formed in a human atherosclerotic plaque that had incorporated a mural thrombus. Immunohistochemistry was performed on paraffin sections as described under materials and methods. (**A**) Immunostaining of endothelial cells by CD31; arrows indicate examples of positive endothelial cells. (**B,C**) Two examples of immunostaining of TAFI with a polyclonal IgG, arrows indicate presence of TAFI. Original magnification 400x.

Discussion

Currently, TAFI is primarily seen as an inhibitor of the plasminogen system during fibrinolysis. Yet, there is increasing evidence that TAFI function may not be restricted to fibrinolysis but that TAFI may also act as a regulator of inflammation and as a modulator of the plasminogen system during tissue remodeling and cell migration. In this report we have provided evidence for the involvement of TAFI in the formation of capillary-like tubular structures by hMVECs in a 3D plasma clot matrix. To our knowledge, these results represent the first attempt to investigate the role of TAFI in tissue remodeling processes *in vitro*.

Different groups have recently generated and characterized TAFI knockout mice and corroborated the absence of an overt phenotype [30-32]. In addition, no anomalous response to a number of acute challenges was found for $TAFI^{-/-}$ mice compared to $TAFI^{+/+}$ wild-type mice. However, this lack of a dramatic phenotype is shared with deficiencies of other components of the fibrinolytic system [48-52] and does not necessarily mean that TAFI does not fulfil a physiologic role. Supporting this notion, te Velde et al. [32] have shown that $TAFI^{-/-}$ mice have impaired wound healing and abnormal keratinocyte migration. Swaisgood et al. [31] combined TAFI deficiency with a plasminogen heterozygous deficiency background demonstrating that TAFI regulates the functions of the plasminogen system both in fibrinolysis and in cell migration *in vivo*. Moreover, it should be noted that during *in vivo* tissue remodeling a functional overlap between the functions of the plasminogen/uPA system and of the MMP system occurs [53]. It has been elegantly shown that wound healing is impaired both in plasminogen-deficient mice and in wild-type mice treated with the MMP inhibitor galardin. Complete arrest of wound healing is only achieved when plasminogen-deficient mice were treated with a MMP inhibitor [53].

On the basis of present knowledge, different mechanisms by which TAFI may play a role in our *in vitro* model of capillary-like tube formation can be envisaged. First, in this model the plasminogen/uPA system localizes the proteolytic activity to specific sites on the cell surface, facilitating matrix degradation and the invasion into the matrix. Therefore, TAFI might inhibit tube formation by removing carboxy-terminal lysines in the plasma clot matrix, preventing the upregulation of plasminogen activation in the matrix and in this way decreasing proteolysis. This mechanism fits with our observations as increasing TAFI or CPB concentrations in the matrix impaired tube formation and decreased proteolysis of the matrix (decrease in FbDP

release). Adding of a TAFIa inhibitor (PCI) or decreasing TAFI concentration in the matrix resulted in the oposite effect that is, the acceleration of tube formation and of matrix proteolysis.

Second, it has been shown previously that the treatment of cells with pancreatic CPB [23,54-56] and with TAFI [29] results in a striking inhibition of plasminogen binding to cells. This binding relies on cell surface receptors which have as common characteristcs their relatively low affinity (Kd~1 μmol/L), high density (10^4 to 10^7 sites/cell) and requirement of free lysine binding sites of plasminogen. To investigate whether TAFI could be involved in the regulation of these receptors we pretreated the hMVECs with CPB before seeding them on top of the plasma clot matrix. Our results show that pretreatment of the cells efficiently inhibits tube formation and downregulates proteolysis suggesting that the modulation of the cell-associated functions of the plasminogen system by TAFI occurs on several levels.

Third, impaired wound healing with a decreased rate of keratinocyte migration has been found in plasminogen-deficient mice [52] and recently a disturbed keratinocyte migration was also reported in $TAFI^{-/-}$ mice [32]. Decreasing TAFI concentration in the plasma clot matrix in our model also altered the magnitude of tube formation and the morphology of the capillary structures formed suggesting alterations of the migration pattern of the hMVECs. In the wound assay, the migration of hMVECs was inhibited in a dose-dependent manner by pancreatic CPB, under the same conditions as used for the tube formation model. The plasminogen/uPA system (uPAR, uPA and plasmin) proved to be involved during the migration of hMVECs, under these conditions as well. In agreement with the *in vivo* results from $TAFI^{-/-}$ mice [31,32], TAFI seems to be able to modulate cell migration.
Finally, modulation of the binding of plasminogen and uPA to cell-surfaces or the proteolysis of cell surface receptors that change the internalization, may cause the activation of signal transduction pathways in the cell [57]. We observed a decrease in the accumulation of uPA in the conditioned medium when hMVECs were pretreated with pancreatic CPB. Although these results are preliminary it has been previously shown that uPA-induced monocyte adhesion requires a carboxy-terminal lysine in a pathway in which cAMP-dependent signal transduction is involved [58].

It should be noted that our results are confined to neovascularization in a plasma clot matrix. We can best perceive this *in vitro* system as a model of wound healing angiogenesis and it can be compared to pathological

conditions such as the neovascularization of a thrombus incorporated in an atherosclerotic plaque. Indeed, TAFI was clearly present both in the matrix and in the endothelial cell lining of the newly formed microvessels in the thrombus that we analysed by immunohistochemistry.

In the *in vitro* model of capillary-like tube formation, the 3D plasma clot matrix was prepared by the addition of thrombin to plasma. Therefore, one may suppose that thrombin and/or thrombin/thrombomodulin were responsible for TAFI activation in this model. The complex between thrombin and thrombomodulin, the endothelial cell receptor of thrombin, is usually depicted as playing a major role in TAFI activation, while the physiological role of plasmin-mediated TAFI activation remains limited. On the other hand, in a cellular environment plasmin-catalyzed activation of TAFI can be stimulated by glycosaminoglycans [4] (15-fold), but the catalytic efficiency of plasmin remains lower than that of thrombin/thrombomodulin. Some characteristics of the assay set-up argue against the idea of thrombin-dependent TAFI activation. Namely, after the plasma clot matrix was prepared it was incubated for 24 hours at 37^{o}C. This will inactivate thrombin and due to the temperature sensitivity, any TAFIa will be readily inactivated, as well. Therefore, it seems more likely that the activation of TAFI in this model might be mediated by plasmin, but this has to be further explored.

In conclusion, our results provide evidence that TAFI is a skilfull modulator of the cellular functions of the plasminogen/uPA system. TAFI regulates at several levels the fine tuning of capillary tube formation and of matrix proteolysis by controling the upregulation of plasminogen binding to the plasma clot matrix and to the cell surface and by controling the migration of hMVECs.

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THESIS | MODULATION OF THE PLASMINOGEN SYSTEM BY THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI)

GENERAL DISCUSSION

The haemostatic balance

Physiological haemostasis is an exquisitely controlled process, essential for blood vessel patency. Haemostasis requires the fast formation of an impermeable platelet and fibrin plug at the site of injury but also demands the local restriction of the active procoagulant substances to the site of injury. The platelet plug rapidly covers the exposed subendothelium and creates the ideal surface for the amplification of thrombin generation, as well as a physical barrier between the active procoagulant substances and the circulating blood [1]. Simultaneously, the fibrinolytic system reacts by binding plasminogen and tissue plasminogen activator to fibrin, the target substrate of plasmin. The localisation of proteolytic and inhibitory activity to this environment allows for selective protection and/or inhibition of the active components, resulting in the efficient regulation of fibrinolysis (*reviewed in* [2]). Cell surfaces also regulate the binding of the components of the plasminogen system by the expression of a variety of cell surface receptors, which are implicated in the spatial organisation, the activation and the inhibition of fibrinolytic components. Plasminogen receptors are characterised by their low affinity, high density and ubiquitous distribution (*reviewed in* [3]) and provide a tool for localised proteolytic activity. Accordingly, the plasminogen/plasmin system is associated with processes, in which pericellular fibrinolysis is required for extracellular matrix degradation and in cell migration.

Thrombin activatable fibrinolysis inhibitor (TAFI) bridges the coagulant and fibrinolytic systems (Chapter 1, *reviewed in* [4-8]) since it is one of the many substrates of thrombin (*reviewed in* [9]) and when active inhibits the amplification of plasminogen activation, thus inhibiting fibrinolysis. Carboxy-terminal basic amino acids (C-terminal lysines and arginines) that are so critical for the upregulation of plasminogen activation [10,11] also mediate other protein-protein interactions. Consequently, these C-terminal residues and TAFI fulfil additional roles in inflammation, vascular tone and cellular migration [12-17].

This chapter encloses the general conclusions provided in this thesis along with a discussion of our results and the literature in an attempt to provide a global view of the current path of TAFI research.

TAFI determination in plasma

In this thesis we have studied the determination of TAFI levels and activity in plasma, which may be useful for the clarification of the pathophysiological mechanisms of TAFI and the evaluation of the diagnostic value of TAFI as a cardiovascular risk indicator. Until recently the migration of the activation peptide of TAFI during SDS-PAGE was not clear, as the peptide cannot be stained in standard staining procedures. In Chapter 2 [18], the migration behaviour of the activation peptide of TAFI during SDS-PAGE was established along with its detection by Western blotting. This knowledge was used to characterise the reactivity of monoclonal anti-TAFI antibodies against either TAFIa or the activation peptide of TAFI. New immunological methods that use these monoclonal antibodies have been, in the meanwhile, developed and constitute a helpful asset for measuring specific fractions of TAFI (e.g. TAFI, TAFIa, TAFI activation peptide) [19]. These assays provide a way to track TAFI activation in both *in vitro* and *in vivo* experimental set-ups. They may also have value in assessing the role of TAFI as a cardiovascular risk indicator but this has still to be established in clinical and epidemiological studies. Until now only one study has been performed concerning the elimination of TAFI and TAFIa from the circulation [20] little information is available regarding the fate of different TAFI fragments (e.g. TAFI activation peptide) *in vivo*. This may pose a problem when attempting to draw conclusions from epidemiological studies, but ultimately such studies will provide valuable information regarding *in vivo* TAFI activation along with the fate of the TAFI fragments following activation.

TAFI variability in plasma – Functional activity

The function of TAFI in fibrinolysis is derived from its ability to cleave C-terminal lysine residues from partially degraded fibrin. A functional TAFI activity determination may bring further insight into the anti-fibrinolytic performance of TAFI in different individuals. Therefore, a new clot lysis-based assay for the determination of the TAFI functional activity in plasma was developed (Chapter 4, [21]). Such a functional assay should be able to trace abnormal molecules with altered activation or inactivation kinetics and/or with diverse activity. Using this assay the TAFI functional variants (TAFI Thr325Ile), which differ slightly in concentration were found to be similar in activity. However, when the activity was corrected for the amount of TAFI protein in each individual an increased specific activity was demonstrated in

individuals expressing the more stable TAFI variant, TAFI 325Ile (Chapter 4, [21]), in agreement with findings with recombinant proteins [22]. This demonstrates the ability of our assay to detect functional differences in TAFIa activity. Moreover, combining the information obtained from different assays, which evaluate both TAFI concentration and TAFI functional activity, may help to further characterise TAFI heterogeneity. In addition, the inactive fragments of TAFI (TAFIai, activation peptide or proteolytic fragments) do not interfere in the determination and the activity measured is specific to the natural substrate of TAFIa during fibrinolysis. Nevertheless, it is important to realise that the assay determines the functional activity of maximally activated TAFI in a plasma sample and that the measurement represents a composed determination of TAFI concentration and activity and of the activity of TAFIa under fixed assay conditions.

TAFI variability in plasma – Issues with assays

The plasma concentration of TAFI was shown to vary considerably in the human population and this variability is poorly explained by environmental factors (*reviewed in* [23]). A strong association was reported between TAFI levels and TAFI gene polymorphisms suggesting a genetic background [24]. In Chapter 3 [25], we established that a lower antibody reactivity towards the TAFI Ile-325 variant of the functional SNP 1040 C/T (Thr325Ile) resulted in an overestimation of the magnitude of the association between TAFI levels and genotypes comprising this SNP. This was also established by Gils *et al.* [26] who developed two distinct ELISAs using specific monoclonal combinations with different immunoreactivity towards the 1040 C/T TAFI SNP (Thr325Ile). In addition, we demonstrated a true association between TAFI levels and TAFI gene polymorphisms by using artefact-free assays (Chapter 3, [25]). Subsequently, the contribution of the TAFI gene variability to the variation in TAFI levels was studied by detailed haplotype analysis (-2599C/G, -2345 2G/1G, -1690A/G, -1102G/T, -438G/A, 505A/G (Ala147Thr), 1040C/T (Thr325Ile), 1542C/G and 1583T/A) and was found to explain only up to 25% of TAFI level variability [27]. This also implies that the remaining variability in TAFI levels ought to be the result of additional factors, some of which might still be assay-related.

Until now, the epitopes of the antibodies used for the majority of the TAFI antigen assays have not been characterised and most assays determine total antigen (Chapter 1) although different reactivities have been observed toward different TAFI fragments (Chapter 2, [18]) [26,28]. Therefore,

besides variable reactivity towards different TAFI genotypes, a variable recognition of TAFI fragments can also contribute to the observed TAFI antigen differences. These fragments can certainly be expected to appear in variable amounts in patients in whom coagulation and fibrinolysis have been systemically activated.

Another problem regarding TAFI assays has to do with the standardisation of the various assays. Commonly used calibrators consist of pooled plasma or purified preparations, which are often poorly characterised and in which the TAFI concentration and activity decay are influenced by the particular haplotype combination in the calibrator (Chapter 3, [25]).

TAFI variability in plasma – Gene expression control

Current knowledge provides some additional factors that might further elucidate the assay-independent variability in TAFI levels. Recently the regulation of mRNA stability was revealed to constitute a mechanism for controlling gene expression [29]. The intrinsic stability of TAFI mRNA in HepG2 cells decreased after combined administration of IL-6 and IL-1 [30]. Hence, the regulation of the mRNA transcript stability in relation to the location of alternative polyadenylation sites in the 3'UTR of the TAFI gene was investigated [31]. The regulation of the TAFI gene expression mediated by cytokines took place through the modulation of transcript stability and polyadenylation site selection. Thus, this variable transcript stability may contribute to the relative abundance of TAFI in the presence of specific stimuli.

Moreover, it has been suggested that TAFI is a positive acute-phase reactant [23,32,33], which creates the possibility that the TAFI gene expression, and particularly TAFI transcription, may be under the control of inflammatory stimuli. In addition, sex hormones seem to play a role in TAFI gene expression [24,34-37]. Therefore an additional mechanism for the regulation of TAFI gene expression may involve the molecular structure of the TAFI promoter or the binding of specific transcription factors although this remains to be elucidated. In fact, a C/EBP-binding site between position -53 and -40 has been identified in the promoter of the TAFI gene and elimination of this site strongly decreased the level of promoter activity in HepG2 cells but did not abolish it completely [38]. This can be explained either by a lower level of promoter activity without the involvement of C/EBP or by the presence of other, less potent, C/EBP-binding sites in the

TAFI promoter. Inspection of the TAFI 5'-flanking region revealed additional potential C/EBP-binding sites [38] some of which are located in the proximity of putative HNF-3 binding sites (TFSEARCH [39]). HNF-3 and C/EBP sites have been described as mutually affecting each other in the IL-6-induced fibrinogen β expression [40]. Interestingly, the promoter region of the C-reactive protein and of fibrinogen α genes, which are also acute phase proteins, enclosed a putative HNF-3 site located adjacent to IL-6 responsive C/EBP elements as well (TFSEARCH). It is well established that C/EBP acts as a critical mediator of immune and inflammatory responses including the acute phase response [41] and this could constitute another mechanism regulating the expression of the TAFI gene. Furthermore, a functional glucocorticoid response element has been identified in the TAFI promoter close to the C/EBP-binding site [38]. Glucocorticoids are required for maximal stimulation of many acute phase genes indicating another possible mechanism by which the transcription of the TAFI gene is activated during inflammatory processes.

TAFI as a risk factor for venous and arterial thrombosis

The hypothesis that TAFI constitutes a risk for thrombotic disorders seems plausible in view of its role as a link between coagulation and fibrinolysis. However, it is well established that the aetiology of venous versus arterial thrombotic disease presents important differences, which may influence the relevance of the TAFI pathway in these two types of thrombosis.

Deep-vein thrombosis (DVT) usually originates in venous valve pockets, within which flow separation results in recirculation [42]. The outcome is a thrombus with a laminar structure consisting of layers of platelets, leukocytes and fibrin, where the main red cell mass is present [43]. In the laminar structure of venous thrombi the platelet-rich layers release, upon activation, the coagulation and fibrinolytic factors present in their α-granules (e.g. FVIII, FXI, PAI-1 and TAFI). Thus, these platelet-rich layers effectively amplify the procoagulant signal driving thrombin generation into the propagation phase. This large-scale thrombin generation will in turn increase TAFI activation, further stabilising the thrombus in these areas by making them resistant to fibrinolysis.

One of the earliest studies focusing on the role of TAFI in venous thrombosis was the LETS study (Leiden Thrombophilia Study). In this case-control study of patients with a first deep-vein thrombosis TAFI antigen levels were

determined [37] using an artefact-free antigen assay (Chapter 1, Chapter 3 [25]). Patients with TAFI levels above the 90th percentile of the controls had a two-fold higher risk of venous thrombosis. In this same study, elevated levels of TAFI increased the risk of thrombosis in individuals with elevated circulating factor VIII concentrations whereas elevated TAFI levels did not increase the risk in factor V Leiden carriers. Another study focussed on the role of TAFI levels as a risk factor in factor V Leiden carriers with venous thromboembolism [44], by determining TAFI with an activity-based assay. Elevated TAFI and factor VIII levels resulted in an increased risk of venous thromboembolism in factor V Leiden carriers. These clinical studies corroborate earlier findings, which indicate a role for the intrinsic coagulation system in the activation of TAFI through the generation of high quantities of thrombin [45-49].

Up to the present only one prospective, multicentre cohort study has investigated the role of TAFI in the recurrence of venous thromboembolism (Austrian Study on Recurrent Venous Thromboembolism, AUREC) [50] by determining the TAFI antigen levels (American Diagnostica, see Chapter 1). A high TAFI level (above the 75th percentile in patients) was associated with a 2-fold higher risk of recurrent venous thromboembolism, though we should keep in mind that this TAFI antigen assay is not well characterised regarding genotype-related artefacts.

The effect on TAFI levels of different heparin regimens, which influence the thrombin generation, was examined in a randomised trial for venous thromboembolism (chromogenic assay, see chapter 1) [51]. The authors suggest that the significant reduction in TAFI levels found in different heparin regimens was related to a more effective regulation of thrombin generation by some of the heparins tested. However, if this were the case a lower thrombin concentration would result in a decrease in TAFI activation and therefore in a relative increase in the TAFI level in plasma.
Finally, the influence of the genetic variation of TAFI gene expression (TAFI genotype and haplotype) on the risk of venous thrombosis was also investigated. However, the results are rather conflicting as some studies found a genotype effect [52] while others found only a trend [53,54] or no effect of the genetic component of TAFI [55-57].

In conclusion, the current data support a contribution of elevated TAFI antigen levels to the risk of venous thrombosis, though larger studies are needed to confirm this association and to clarify the effect of the genetic

component of TAFI as well as the relation of TAFI to other known risk factors.

Arterial thrombosis usually occurs under high shear stress and is accompanied by significant modification or damage to the vessel wall (atherosclerosis) leading to the formation of a typical thrombus consisting of a platelet-rich "head" followed by a fibrin-rich "tail" [42]. The role of platelets in arterial thrombosis is certainly different from that in venous thrombosis. During arterial thrombosis the platelet plug constitutes the first response to injury while the subsequent decrease of blood flow promotes the formation of fibrin. TM redirects the activity of thrombin towards TAFI activation. The TAFI activation promotes the stabilisation of the fibrin network and possibly prevents embolisation.

The relationship between TAFI and arterial thrombosis has also been examined using both antigen- and activity-based assays, previously described in Chapter 1. Discussing the role of TAFI in arterial thrombosis is intricate in view of the controversial results previously published. Some of these studies found TAFI to be a risk factor for arterial disease [32,58-66], whereas others found it to be protective [67-69] or to have no effect [70-76]. These discrepancies might arise from the lack of proper characterisation of the assays used as was discussed above and as shown by the results of the Prospective Epidemiological Study of Myocardial Infarction (PRIME study) [68,75,77]. Therefore, here we will restrict our discussion to clinical studies, which have been performed with antigen assays, free from genotype-related artefacts (Chapter 1, Chapter 3 [25] and [19,26,27]) or with activity-based assays (Chapter 4 [65] and [32,60-62,72,73]). The studies with various set-ups (case-control, prospective) were conducted in patients with either a cerebral (ischaemic stroke, IS) or a cardiac (myocardial infarction, MI; angina pectoris, AP; coronary artery disease, CAD) ischaemic event.

Two case-control studies investigated the association between TAFI levels and the risk of ischaemic stroke [60,65]. In both studies elevated TAFI resulted in an increased risk of ischaemic stroke (TAFI levels in patients>120%, OR 5.7; 95%CI 2.3-14.1 and TAFI levels in patients in the highest quartile, OR 4.0; 95%CI 1.6-9.8, respectively). Moreover, TAFI levels remained elevated in ischaemic stroke patients even 3 months after the event [65] whereas CRP levels decreased after 3 months, suggesting that the elevation of TAFI was not due to the acute phase response.

Silveira *et al.* [32] determined TAFI levels in patients requiring coronary artery bypass grafting (CABG) due to stable angina pectoris. They showed that TAFI levels declined from their preoperative level three days after surgery but rose in relation to the preoperative level six days after surgery. These variations may be related to the consumption of TAFI after surgery and to increases in TAFI gene expression during the inflammatory stage. Though TAFI was found to be an acute phase protein very little is known about its fluctuation upon the onset of inflammation and the relation between the inflammatory response profile of TAFI and other acute phase proteins, such as fibrinogen, α_1-antitrypsin and CRP. The role of TAFI levels in acute CAD [62] was also investigated and again a significant increase in risk with high TAFI levels (TAFI levels in patients>126%, OR 3.5; 95%CI 1.3-8.7) was encountered. On the other hand, no difference in TAFI levels was found either in a case-control acute MI study [72] or in a study aiming to predict reperfusion after acute MI [73] whereas a significant increase in TAFI levels was observed in young patients with acute MI [61]. In the PRIME study (MI and angina pectoris) no association between TAFI levels and the risk of coronary heart disease was found.

A role for TAFI in arterial thrombosis might arise from the differential expression of thrombomodulin (TM), which is modulated through a variety of factors [78]. TM is down regulated in the presence of oxidised LDL, increased shear stress conditions and under inflammatory conditions where oxidation of methionine 388 of TM occurs, conditions which are typical of the atherosclerosis process and thus are somehow involved during arterial thrombosis. This down-regulation may result in the inhibition of APC pathway while TAFI activation is preserved. This together with an enhanced transcription of the TAFI gene under inflammatory conditions may lead to an increased risk of developing arterial thrombosis.

Modulation of the plasminogen system by TAFI

At present, any thrombolytic therapy regimen relies on the activation of the fibrinolytic system in the patient by the administration of plasminogen activators (PAs) [79]. These PAs convert the precursor plasminogen into plasmin, the active enzyme responsible for the proteolytic degradation of the fibrin fibres of the thrombus. It follows that the efficiency of thrombolysis is influenced by the properties of the PA, the thrombus structure, the supply of plasminogen and PA to the boundary and interior of the thrombus and by the availability of the inhibitors that regulate the activity of the fibrinolytic

system. These inhibitors are directed towards the inhibition of the PAs (PAI-1), the direct inhibition of plasmin (plasmin inhibitor, previously named α_2-antiplasmin and to a lesser extent α_2-macroglobulin) and the inhibition of the cofactor activity of partially degraded fibrin on plasminogen activation (TAFI). Up to now, tPA has been the PA most widely used for investigating the effect of TAFI on fibrinolysis both *in vitro* [46,80-87] and *in vivo* [88-93]. However, it still remains unclear whether TAFI contributes to the sub-maximal efficacy observed during thrombolytic therapy. In Chapter 6 [94] we investigated the inhibitory effect of TAFI in an internal plasma clot lysis model mediated by a variety of PAs, which represent all major classes of currently available thrombolytic drugs. In such a model, plasminogen and the PA are distributed throughout the plasma clot. TAFIa inhibited clot lysis mediated by each PA in a PA concentration-dependent manner. The effect of TAFI was small at low PA concentrations, which we attributed to the restricted stability of TAFIa. The maximal TAFIa-related inhibition varied for different PAs but was not related to the fibrin specificity of the PA. As TAFIa eliminates the newly formed binding sites for plasminogen and plasmin on fibrin, it might be able to modulate plasmin activity. It is known that fibrin-bound plasmin becomes more resistant to inactivation by plasmin inhibitor (PI) [95,96]. We studied the role of PI in the mechanism of TAFIa retardation of clot lysis (Chapter 6, [94]). In our model, PI did not play a significant role in the inhibition of plasma clot lysis by TAFI. Recently, TAFIa was shown to eliminate the protection of plasmin from PI by cleaving the exposed C-terminal residues on partially degraded fibrin, in a purified system [97]. This apparent discrepancy might result from the differences between the two systems used.

In addition, we investigated the fibrinolytic efficacy of three PAs (Tenecteplase, Amediplase and scu-PA) in external plasma clot lysis models examining the inhibitory effect of TAFI as well (Chapter 7). External plasma clot lysis resembles more thrombolytic therapy as the PA reaches the clot after its formation. TAFIa also displayed an inhibitory effect in external lysis, when thrombomodulin was present. This effect was again dependent on the type and concentration of the PA used. The inhibitory effect of TAFIa was high at low PA concentration and decreased at increasing PA concentrations.

The TAFIa effect became negligible in the therapeutic range, both in the internal and external plasma clot lysis model (Chapter 6 and 7). Our findings show that the loss of the TAFIa effect coincides with plasminogen

and PA concentrations at which a high plasmin concentration is achieved. This may be the outcome of the inhibition of TAFI and TAFIa by proteolytic cleavage by plasmin or arise from the loss of fibrin specificity that makes plasminogen activation less dependent on the availability of C-terminal lysines on fibrin. This is in agreement with previous findings [80,85,98]. These results suggest that the role of TAFI during optimal and successful thrombolytic therapy is probably low. Nevertheless, depending on the thrombus composition and architecture, haemodynamic conditions and distinct physiological interactions of plasminogen and PAs in the vascular environment, it is conceivable that the local PA concentration does not always reach optimal levels. This creates conditions, in which TAFI might affect the lysis rates during thrombolytic therapy.

The role of the plasminogen system in cardiovascular disease is not restricted to the degradation of fibrin as this system is also involved in the degradation of other matrix proteins, the activation of matrix metalloproteinases, the regulation of growth factor and chemokine pathways and in directed cell migration (*reviewed in* [99]). Plasminogen is activated to plasmin during wound healing [100] and a delayed reepithelisation of cutaneous wounds is observed in plasminogen-deficient mice [101]. In physiological plasminogen activation tPA seems to function mostly intravascularly, whereas uPA acts predominantly in extravascular spaces as a major player in cell migration and matrix remodelling [102]. In Chapter 8, we investigated the role of TAFI during *in vitro* capillary tube formation by human microvascular endothelial cells (hMVECs) in a 3D plasma clot matrix. In this *in vitro* model of wound healing, the plasminogen system plays a pivotal role and activation is mainly mediated by uPA [103]. TAFI is capable of preventing the upregulation of plasminogen activation not only on partially degraded fibrin but probably also on cell surfaces (shown with pancreatic carboxypeptidase B treatment, which is homologous to TAFIa [104-108]). We showed *in vitro* that this ultimately leads to the inhibition of cellular proteolysis and to a decreased and/or altered cell migratory response. This effect of TAFI on cellular migration was observed not only in our *in vitro* capillary tube formation experiments (Chapter 8) but also in an *in vivo* model of wound healing [16] and in a thioglycollate model of peritoneal inflammation [15]. These results corroborate the role of TAFI as a broad modulator of the functions of the plasminogen system.

Cross-talk between systems

During the initiation phase of coagulation the availability of thrombin substrates and of its cofactors directs the initial burst of thrombin activity affecting thrombus architecture and composition [9]. Thrombin substrate recognition involves the direct interaction between the substrate and thrombin's exosites with or without the assistance of a cofactor, which increases considerably the efficiency of substrate cleavage. For substrate reactions assisted by cofactors, the reaction is rather inefficient in the absence of the cofactor and therefore unlikely to take place *in vivo* [9]. Hence it is believed that the complex between thrombin and thrombomodulin (TM) constitutes a physiologic activator of TAFI in the initiation phase of coagulation. The thrombin/TM complex is also the physiological activator of protein C [109]. This is important in the context of an injury or inflammatory stimulus where the cell-surface expression of TM is abrogated by various pathways [78]. Subsequently, the suppression of TM expression leaves thrombin activity uncontrolled resulting in the amplification of the inflammatory stimulus. Under these conditions, i.e. low TM, protein C activation is hampered while TAFI activation is relatively preserved [110], stabilising the thrombus at the site of injury and possibly also down-regulating inflammation [12-15]. Moreover, the ability of TAFIa to avoid precocious thrombus dissolution and thus prevent delayed rebleeding has provided an explanation for the bleeding tendency of factor XI-deficient individuals. The failure to produce high thrombin concentrations via a factor XI-dependent way was shown to decrease TAFI activation and to downregulate fibrinolysis in these individuals [111,112]. In fact, TAFI activation in the presence of TM, which requires only low thrombin concentrations, was able to correct the premature lysis of clots from factors X-, IX-, VIII-, and XI-deficient plasmas *in vitro* [84] (*reviewed in* [6,7]). Both tissue transglutaminases (TG) and FXIIIa catalysed the cross-linking of TAFI and TAFIa to fibrin [113]. The binding depended on the concentration of TG or FXIIIa and on the availibility of calcium. TAFI was cross-linked to a similar extent as was α_2-antiplasmin, suggesting that the incorporation has biological significance. Interestingly, the cross-linking sites were located both in the TAFI activation peptide and in TAFIa suggesting that TAFIa remains associated with fibrin, which might increase its conformational stability and alter TAFI activation and inactivation kinetics significantly.

Glycosaminoglycans can enhance activation of the complement cascade as well as the activation of TAFI by plasmin [114], which in turn can inhibit

some components of the complement system [12-15]. Recently, it was shown that the activation of TAFI mediated by plasmin occurred during the final phase of internal plasma clot lysis [115]. In such a system this second burst of TAFI activity did not influence fibrinolysis as it occurred in the end stage of lysis. However, in the *in vivo* situation where external lysis takes place activation of TAFI by plasmin may occur and may have physiological relevance both in fibrinolysis and in inflammation management. If it is established that plasmin has the ability to modulate TAFI activation *in vivo*, the downregulation of inflammation by TAFIa may accompany the proteolytic activity of the plasminogen system. CPN, the other carboxypeptidase B, present in the circulation as an active enzyme that is also able to downregulate inflammation [116] fulfils a different role from TAFIa in the circulation.

Under normal conditions, the vasculature remains intact and the haemostatic balance is maintained. Howerver, chronic exposure of the vascular bed to a variety of subtle stimuli slowly but surely devises a state of endothelial dysfunction. Such a endothelial dysfunction state is often accompanied by increased vascular permeability followed by extravasation of plasma proteins and may result in a vicious circle where inflammation-induced coagulation leads to coagulation-induced inflammation [117].

Currently the understanding of the role of TAFI in the cross-talk between coagulation, complement system, fibrinolysis, pericellular fibrinolysis, atherosclerosis and inflammation is rather limited. The intricacy of TAFI provides additional challenges when investigating the role of TAFI in these processes. At present it is becoming clear that the functions of TAFI in the vasculature go beyond the inhibition of fibrinolysis.

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THESIS

MODULATION OF THE PLASMINOGEN SYSTEM BY THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI)

SUMMARY / SAMENVATTING

English summary

Haemostasis is an exquisitely controlled process, essential for blood vessel patency. Haemostasis requires the fast formation of an impermeable platelet and fibrin plug at the site of injury but also demands the local restriction of the active procoagulant substances to the site of injury. The platelet plug rapidly covers the exposed subendothelium and creates the ideal surface for amplification of thrombin generation, as well as a physical barrier between the active procoagulant substances and the circulating blood. Simultaneously, the fibrinolytic system reacts by binding plasminogen and tissue plasminogen activator to fibrin, the target substrate of plasmin. The localisation of proteolytic and inhibitory activity to this environment allows for selective activation, protection and/or inhibition of the active components, resulting in an efficiently regulated fibrinolytic system. Cell surfaces also regulate the binding of the components of the plasminogen system by the expression of a variety of cell surface receptors, which are implicated in the spatial organisation, the activation and the inhibition of these fibrinolytic components. Thrombin activatable fibrinolysis inhibitor (TAFI) bridges the coagulant and fibrinolytic systems since it can be activated to become TAFIa by thrombin formed via the coagulation cascade and TAFIa inhibits the amplification of plasminogen activation, thus inhibiting fibrinolysis. The mechanism of action of TAFI is related to the second phase of plasminogen activation. In this phase plasmin cleaves fibrin and generates carboxy-terminal lysine residues in the fibrin network, which represent additional binding sites for plasminogen. As a result of additional plasminogen binding, plasminogen activation and fibrinolysis are strongly accelerated. TAFIa, being a carboxypeptidase B, eliminates carboxy-terminal terminal lysine and arginine residues thus blocking the upregulation in the plasminogen activation.

The work presented in this thesis was structured in three sections. The first section deals with some biochemical properties of TAFI while the second section describes the assessment of TAFI assays along with the development, validation and implementation of new methodology for the functional determination of TAFI in plasma samples. Finally, the third section examines the involvement of TAFI in the modulation of the plasminogen system during fibrinolysis and during pericellular proteolysis.

In **Chapter 1** the current knowledge in the TAFI field is reviewed and the characteristics of the currently available TAFI assays are considered in detail.

The activation peptide of TAFI is heavily glycosylated with the carbohydrate content accounting for about 20% of the total mass of the zymogen. However, until recently the migration of the activation peptide of TAFI during SDS-PAGE was not clear. In this thesis (**Chapter 2**), the migration behaviour of the activation peptide of TAFI during SDS-PAGE was established along with its detection by Western blotting. In addition, we used this knowledge to characterise the reactivity of monoclonal anti-TAFI antibodies against either TAFIa or the activation peptide of TAFI, facilitating the development of assays appropriate for tracking TAFI activation in vitro and in vivo.

In **Chapter 3** we evaluated several assays for the determination of TAFI in plasma. The results in this chapter demonstrate a variable immunoreactivity of some antibodies towards TAFI Thr325Ile isoforms (TAFI 1040C/T SNP). The recognition of the TAFI 325Ile functional variant by these antibodies is impaired, leading to an underestimation in the determination of TAFI antigen levels and to a fictitious amplification of the genotype-related variation of TAFI concentration. Still, we showed a true association between TAFI levels and TAFI gene polymorphisms when using artefact-free assays. **Chapter 4** covers the development and validation of a new TAFI functional activity assay, based on the retardation of plasma clot lysis by TAFIa. The TAFI-related retardation of clot lysis was determined in a group of healthy individuals and found to correlate with TAFI antigen level while the specific antifibrinolytic activity of TAFI was associated with the - 438A/G and 1040C/T TAFI genotypes. Thereafter, in **Chapter 5** we addressed the role of TAFI functional activity levels as well as TAFI genotype distribution in relation to ischaemic stroke. In a case-control study (COCOS study) no association was found between TAFI genotype or haplotype and the risk of stroke. However, increased TAFI functional activity was associated with a higher risk of ischaemic stroke even after adjustment for potential confounders.

TAFI is nowadays regarded as a potent inhibitor of fibrinolysis but its effect on the efficiency of thrombolysis which is influenced by the properties of the plasminogen activator (PA), the thrombus structure and the supply of plasminogen and PA to the boundary and interior of the thrombus, is not fully resolved. Therefore, in **Chapter 6** we investigated the inhibitory effect of TAFI in an internal plasma clot lysis model mediated by a variety of PAs, which represent all major classes of thrombolytic drugs. TAFI inhibited clot lysis mediated by each PA in a PA concentration-dependent manner. In addition, in **Chapter 7** we investigated the fibrinolytic efficacy of three PAs in external plasma clot lysis models, examining as well the inhibitory

effect of TAFI. TAFIa displayed an inhibitory effect in external lysis, when thrombomodulin was present. This effect was dependent on the type and concentration of the PA used. The inhibitory effect of TAFIa was high at low PA concentration and decreased with increasing PA concentrations. Both in the internal and external plasma clot lysis model the TAFIa effect became negligible in the therapeutic range. Our findings show that the loss of the TAFIa effect coincides with plasminogen and PA concentrations at which a high plasmin concentration is achieved. This may be the outcome of the inhibition of TAFI and TAFIa by proteolytic cleavage by plasmin or arise from the loss of fibrin specificity that makes plasminogen activation less dependent on the availability of C-terminal lysines on fibrin. There is growing evidence suggesting that TAFI behaves as a broad modulator of the plasminogen system. In **Chapter 8**, we studied the involvement of TAFI in an in vitro model of capillary-like tube formation, a wound-healing angiogenesis model where a 3D plasma clot matrix is employed. Due to the composition of the matrix, the results may be extrapolated to the neovascularisation of a fibrinous exudate in vivo. TAFI modulated in vitro capillary tube formation by inhibiting plasminogen-dependent cellular proteolysis and decreasing and/or modifying endothelial cell migration.

Chapter 9 contains a general discussion of our work in relation to recent developments in TAFI research. Special attention is given to the determination of TAFI in plasma considering the role of different assays and of gene expression control on the variability of TAFI concentration as well as the repercussions to the evaluation of TAFI as a risk factor in venous and arterial thrombosis. Finally, the modulation of the plasminogen system by TAFI is discussed together with its role in the cross-talk between coagulation, complement system activation, fibrinolysis, pericellular fibrinolysis, atherosclerosis and inflammation. The intricacy of TAFI provides additional challenges when investigating the role of TAFI in this cross-talk. At present it is already becoming clear that the functions of TAFI in the vasculature go beyond the inhibition of fibrinolysis.

Nederlandse samenvatting

Hemostase is een uitermate nauwkeurig gereguleerd proces en van groot belang voor het intact en open houden van bloedvaten. Voor hemostase is de snelle vorming van een impermeabele trombocyten- en fibrineplug nodig, maar ook beperking van de actieve stollingsbevorderende factoren tot de beschadigde plek. De trombocytenplug bedekt het blootgelegde subendotheliale weefsel snel en vormt het optimale oppervlak voor amplificatie van trombinevorming en vormt bovendien een fysieke barrière tussen de actieve stollingsbevorderende factoren en het circulerende bloed. Tegelijkertijd reageert het fibrinolytisch systeem door de binding van plasminogeen en weefselplasminogeenactivator (tPA) aan fibrine, het substraat van plasmine. De concentratie van proteolytische en remmende activiteiten op deze plek zorgt voor selectieve activatie, bescherming en/of remming van de actieve componenten en dit resulteert in een efficiënte regulatie van de fibrinolyse. Celoppervlakken reguleren eveneens de binding van componenten van het plasminogeenactivatie systeem door de expressie van verschillende receptoren, die betrokken zijn bij de ruimtelijke organisatie, de activatie en de remming van deze fibrinolytische bestanddelen. De door trombine activeerbare fibrinolyse inhibitor (TAFI) vormt een brug tussen het stollings- en het fibrinolytisch systeem, doordat het geactiveerd kan worden tot TAFIa door trombine dat gevormd wordt in de stollingscascade en TAFIa de amplificatie van plasminogeenactivatie verhindert en zo fibrinolyse remt. Het werkingsmechanisme van TAFI is gerelateerd aan de tweede fase van plasminogeenactivatie. In deze fase wordt fibrine door plasmine gekliefd en ontstaan carboxy-terminale lysine residuen in het fibrine netwerk en deze vormen additionele bindingsplaatsen voor plasminogeen. Als gevolg van de additionele plasminogeenbinding worden plasminogeenactivatie en fibrinolyse sterk versneld. TAFIa is een carboxypeptidase B en verwijdert carboxy-terminale lysine en arginine residuen en blokkeert zo de toename van de plasminogeenactivatie.

Het onderzoek in dit proefschrift is verdeeld in drie secties. In het eerste gedeelte worden enkele biochemische eigenschappen van TAFI bestudeerd, terwijl in het tweede deel TAFI assays worden beoordeeld en de ontwikkeling, validatie en implementatie van een nieuwe methode voor de functionele bepaling van TAFI in plasma wordt beschreven. Tenslotte wordt in het derde deel de betrokkenheid van TAFI bij de modulering van het plasminogeen systeem tijdens fibrinolyse en tijdens pericellulaire proteolyse onderzocht.

In **hoofdstuk 1** wordt een overzicht gegeven van de huidige kennis in het

onderzoeksgebied van TAFI en worden de karakteristieken van de op dit moment beschikbare TAFI assays in detail besproken. Het activatiepeptide van TAFI is sterk geglycosyleerd waarbij de koolhydraat component ongeveer 20% van de gehele zymogeenmassa vormt. Tot voor kort was echter de migratie van het activatiepeptide van TAFI tijdens SDS-PAGE niet duidelijk. In dit proefschrift (**hoofdstuk 2**) werden de migratie eigenschappen tijdens SDS-PAGE van het activatiepeptide van TAFI vastgesteld door de detectie ervan door Western blotting. Daarnaast werd deze kennis gebruikt om de reactiviteit van monoclonale antistoffen tegen TAFIa en het activatiepeptide van TAFI te karakteriseren, hetgeen de ontwikkeling van assays om TAFI activatie in vitro en in vivo te kunnen volgen, vereenvoudigde.

In **hoofdstuk 3** worden verschillende assays voor de bepaling van TAFI in plasma geëvalueerd. De resultaten in dit hoofdstuk tonen een variatie aan van de immunoreactiviteit van enkele antistoffen tegen TAFI Thr325IIe isovormen (TAFI 1040C/T SNP). De herkenning van de TAFI 325IIe functionele variant door deze antistoffen is verminderd en dit leidt tot een onderschatting bij de bepaling van TAFI antigeen concentraties en tot een fictieve toename van de genotype-gerelateerde variatie in TAFI concentraties. Desondanks konden wij een echte associatie aantonen tussen TAFI concentraties en TAFI genpolymorfismen door gebruik van assays zonder deze artefacten. **Hoofdstuk 4** omvat de ontwikkeling en validatie van een nieuwe assay voor de bepaling van de functionele activiteit van TAFI gebaseerd op de vertraging van de lysis van plasma stolsels door TAFIa. De aan TAFI gerelateerde vertraging van de stolsellysis werd bepaald in een groep gezonde individuen en bleek te correleren met TAFI antigeen concentraties terwijl de specifieke antifibrinolytische activiteit van TAFI geassocieerd was met de -438 A/G en 1040 C/T TAFI genotypes. Daarna, in **hoofdstuk 5**, bestudeerden we de rol van de functionele TAFI activiteit in relatie tot ischemische herseninfarcten. In een case-control studie (COCOS studie) werd geen associatie gevonden tussen TAFI genotype of haplotype en het risico op een herseninfarct. Een toegenomen functionele activiteit van TAFI was daarentegen wel geassocieerd met een hoger risico voor een ischemisch herseninfarct, ook na correctie voor potentiële confounders.

TAFI wordt tegenwoordig beschouwd als een krachtige remmer van de fibrinolyse, maar het effect ervan op de efficiëntie van thrombolyse, welke beïnvloed wordt door de eigenschappen van plasminogeenactivator (PA), de structuur van de trombus en toevoer van plasminogeen en PA naar zowel de buitenkant en het binnenste van de trombus, is nog niet

geheel bekend. Daarom hebben we, in **hoofdstuk 6**, het remmende effect van TAFI onderzocht in een intern plasmastolsellysis model, gemedieerd door verschillende soorten PAs die alle grote klassen thrombolytische geneesmiddelen vertegenwoordigen. TAFI remde stolsellysis gemedieerd door elke PA op een PA concentratie-afhankelijke manier. Daarnaast bestudeerden we in **hoofdstuk 7** zowel de fibrinolytische effectiviteit van drie PA's als de remmende invloed hierop van TAFI in een extern stolsellysis model. TAFIa vertoonde een remmend effect in het externe lysis model in aanwezigheid van thrombomoduline. Dit effect was afhankelijk van de soort en de concentratie van de gebruikte PA. Het remmende effect van TAFIa was sterk bij een lage PA concentratie en nam af bij toenemende PA concentraties. Zowel in het externe als het interne plasmastolsellysis model werd het TAFIa effect verwaarloosbaar in de therapeutische range. Onze bevindingen tonen aan dat het verlies van het TAFIa effect samenvalt met plasminogeen en PA concentraties waarbij een hoge plasmine concentratie wordt bereikt. Dit zou het gevolg kunnen zijn van de remming van TAFI of TAFIa door proteolytische klieving door plasmine of zou het gevolg kunnen zijn van het verlies van fibrine-specificiteit dat plasminogeenactivatie minder afhankelijk maakt van de beschikbaarheid van C-terminale lysine residuen op fibrine. Er zijn steeds meer aanwijzingen dat TAFI zich gedraagt als een brede modulator van het plasminogeen systeem. In **hoofdstuk 8** hebben we de betrokkenheid van TAFI onderzocht in een in vitro model van capillair vorming, een wondhelings-angiogenese model waarin een 3D plasma stollingsmatrix wordt toegepast. Door de samenstelling van de matrix zouden de resultaten geëxtrapoleerd kunnen worden naar de neovascularisatie van een fibrineus exsudaat in vivo. TAFI moduleerde de in vitro capillair vorming door remming van de plasminogeen-afhankelijke cellulaire proteolyse en door afname en /of verandering van de migratie van endotheelcellen.

Hoofdstuk 9 bevat een algemene discussie van ons werk in relatie tot recente ontwikkelingen in het TAFI onderzoek. Speciale aandacht wordt gegeven aan de bepaling van TAFI in plasma rekening houdend met de rol van de verschillende assays en van de controle door genexpressie op de variatie in TAFI concentraties evenals de repercussies op de evaluatie van TAFI als risicofactor voor veneuze en arteriële trombose. Tenslotte wordt de modulatie van het plasminogeen systeem door TAFI besproken samen met de rol van TAFI in de "cross-talk" tussen stolling, complement activatie, fibrinolyse, pericellulaire fibrinolyse, atherosclerose en ontsteking.

De complexiteit van TAFI leidt tot nieuwe uitdagingen voor het onderzoek naar de rol van TAFI in deze “cross-talk”. Momenteel wordt al duidelijk dat de functies van TAFI in het vaatstelsel verder gaan dan remming van fibrinolyse alleen.

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Curriculum Vitae

Ana Helena Canas Guimarães was born on the 1st of June of 1976, in Braga, Portugal. In 1992, she achieved the certificate of proficiency in English from the University of Cambridge. She graduated from Escola Secundária Carlos Amarante, in 1994. In the same year she carried out a part-time project as assistant in the chemistry research laboratory at the University of Minho in Braga.

Following highschool graduation she enrolled at the Faculty of Science of the University of Oporto where in 1999 she concluded the Master of Science in Chemistry, which included a specialization in Analytical Chemistry under supervision of Dr. Cosme Moura.

During the following year she acquired practical experience at Akzo Nobel Central Research in Arnhem, The Netherlands working in polymer chemistry and colloid physics under the supervision of Dr. J.G. Batelaan.

Subsequently, in November 2000, she initiated her PhD project at the Laboratory for Physiology at the ICaR, VU University Medical Center in Amsterdam with Prof. Dr. V.W.M. van Hinsbergh as tutor and Dr. D.C. Rijken as supervisor. The work described in this thesis was performed at the Gaubius Laboratory, TNO-PG in Leiden where she was detached to until December 2003 and from then on at the Department of Hematology of the Erasmus MC, University Medical Center Rotterdam. Since January 2005, she works as a Postdoc at the Department of Hematology of the Erasmus MC, University Medical Center Rotterdam, where she continues studying the role TAFI in cardiovascular disease.